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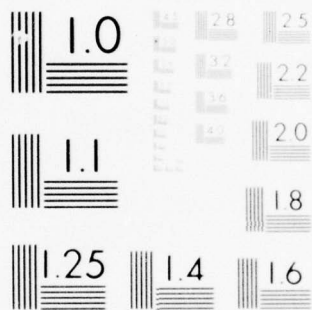
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ADJUVANT EFFECTS ON IMMUNE
RESPONSES TO BIOLOGIC AGENTS

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20 March 1977
(For the Period 1 July 1976 - 30 June 1977)

by
Norman D. Anderson, M.D.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) This report describes the patterns of lymphocyte recirculation in normal animals. Radiokinetic and autoradiographic studies demonstrate that long- lived T and B cells selectively localize within organized lymphatic tissue as they recirculate between blood and lymph. Within the spleen, fluid flow through the open reticular meshwork permits both normal, enzyme-altered, and malignant lymphocytes to move across the splenic white pulp in a normal		

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20. manner so that cellular traffic in this organ probably does not depend upon the same surface recognition mechanisms and cellular motility required for entry into peripheral lymph nodes. Recirculating lymphocytes move through all peripheral nodes and their apparent preferential accumulation within mesenteric nodes can be attributed to the large mass, greater blood flow and higher background antigenic stimulation of these nodes rather than the presence of lymphocyte subpopulations which selectively "home" into gut-associated lymphatic tissue. The correlation between histologic and quantitative measurement of cellular traffic into lymphatic tissues of young animals and the extent of development of high endothelial venules provides further support for the importance of cell surface recognition mechanisms in regulating lymphocyte recirculation. Ultrastructural, autoradiographic, and radiokinetic data are presented which indicate that Cytochalasin A treated-lymphocytes can attach to HEV surfaces but fail to emigrate into lymph nodes. These observations provide the first direct demonstration that the movement of lymphocytes into peripheral nodes can be separated into a recognition-attachment phase followed by active emigration. The possible mechanisms which direct movement of these motile lymphocytes across the HEV wall are now being investigated using our newly developed techniques for studying lymphocyte migration under agarose, and preliminary evidence for a lymphocyte chemotactic factor derived from IgM is presented.

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SUMMARY

This report presents the progress on research supported by contract number DAMD 17-74-C-4095 during the fiscal year of 1977. Morphologic, immunologic, and radiokinetic findings have been carried out to provide new insight into the regulation of lymphocyte traffic in normal, antigen, and adjuvant treated animals. The patterns of lymphocyte recirculation through the spleen and lymph node are described and the biological determinants of this process are analyzed. A new technique for studying lymphocyte chemotaxis in vitro has been developed in this laboratory and preliminary evidence for a chemotactic factor derived from IgM is presented which may offer a new and physiologic means for manipulating immune responses in vivo.

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FOREWARD

This annual progress report is a summary of the research activities carried out by Norman D. Anderson, M.D. (Principal Investigator), Robert Hoffman, B.S. (Associate Investigator) at The Johns Hopkins University School of Medicine, Baltimore, Maryland in collaboration with Major Arthur O. Anderson (Associate Investigator) at the U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland.

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In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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INTRODUCTION

Research Objectives. The goals of this research are to define the biological mechanisms of immunopotential and to utilize this information in developing new adjuvants for augmenting immune responses to marginal vaccines.

Background Information. There is convincing evidence that successful initiation of the immune response depends upon a complex sequence of antigen presentation, collaboration between T-lymphocytes, B-lymphocytes, and macrophages and subsequent cellular proliferation and differentiation which are modulated by suppressor cells, local release of lymphokines and other cell products such as specific antibody (1, 2, 3, 9). Since the effective use of adjuvant agents requires appropriate manipulation of one or more of these events in vivo (3), precise definition of the structural and functional determinants of unmodified immune responses in established lymphatic tissues remains as a prerequisite for understanding the mechanisms of immunopotential.

Organized lymphatic tissue, with its complex cellular populations, reticular meshwork, lymphatic and vascular connections provides an efficient locus for facilitating in vivo immune responses. There is ample evidence indicating that lymph nodes serve as stationary filters which effectively bind and concentrate antigens moving through lymphatics while the spleen plays a similar role in removing antigen from the blood. Studies by Gowans (4) and others have established that immunocompetent T and B lymphocytes continually recirculate between blood and lymph. Since this selective movement of lymphocytes through the spleen and lymph nodes provides a highly efficient means of immunosurveillance, this selective cellular traffic has been regarded as a critical step for initiating immune reactions in vivo. Despite this, there has been prolonged controversy over the mechanisms which regulate cellular traffic in lymphatic tissues. Studies described in our previous progress reports have: (1) provided the first detailed description of the lymph node microvasculature and the local hemodynamic control mechanisms which regulate the distribution of blood-borne lymphocytes within lymph nodes; (2) defined the route of lymphocyte entry into lymph nodes through intercellular spaces in the walls of high endothelial venules; (3) demonstrated that intercellular spaces through which lymphocytes emigrate function as anatomical communications between the blood and lymph so that macromolecules could flow from the node into the HEV lumen and establish a chemotactic gradient; (4) provided morphologic data confirming that lymphocytes actively migrate across the HEV wall showing directional movement from the venular lumen into the nodal parenchyma; (5) established that this sequence of lymphocyte movement into the node can be abrogated by a variety of pharmacologic agents which coat cellular membranes, aggregate microfilaments or disrupt microtubules; (6) indicated that a variety of chemically disparate adjuvants have the common property on augmenting cellular traffic into the regional node; and

(7) provided the first accurate descriptions of lymphocyte traffic patterns in the spleen with clear evidence for efferent lymphatics serving as the major exit pathway for lymphocytes moving out of the splenic white pulp.

These findings have now been complemented by data presented in Project I documenting the presence of fluid wave which facilitates lymphocyte movement into the splenic white pulp and efferent lymphatics. The importance of this fluid flow pattern is emphasized by our radiokinetic and autoradiographic observations presented in subsequent sections of this report which indicate that both normal and non-motile lymphocytes move through the spleen in a nearly identical manner. When this data is combined with our findings on the traffic of enzyme-treated and malignant lymphocytes, it seems quite likely that lymphocyte recirculation in the spleen is not dependent upon the same surface recognition mechanisms or intrinsic cellular motility required for movement through peripheral lymph nodes. Project II describes the sequential pattern of lymphocyte movement through different lymph nodes in both weanling and adult rats. These findings in combination with the data on selective ablation of different lymphatic tissues indicate that the apparent preferential sequestration of recirculating lymphocytes within gut-associated lymphatic tissues probably results from their greater mass and blood flow and not organ specific surface receptors postulated by other investigators. Project III summarizes our in vitro, radiokinetic, autoradiographic, and ultrastructural findings which document that lymphocytes rendered non-motile by Cytochalasin A attach to HEV surfaces but fail to move into lymph nodes. This represents the first direct demonstration that lymphocyte entry into lymph nodes can be separated into a recognition-attachment phase followed by active emigration. Project IV describes our progress in developing a new in vitro method for studying lymphocyte migration under agarose and includes preliminary data demonstrating that a fragment from rat IgM is selectively chemotactic for thoracic duct lymphocytes. Project V briefly outlines data from our combined morphologic, autoradiographic, radiokinetic, and cell traffic studies of the regional nodes draining local injections of antigen or adjuvant which certainly suggest that these methodologies will be most useful in defining the mechanisms and developing new agents for use in immunopotentialiation.

PROJECT I. Lymphocyte Recirculation in the Spleen.

The spleen is the largest lymphoid organ in the body and has been considered to represent a modified lymph node situated astride major blood vessels so that it continually filters the blood and provides the major anatomical site for immune responses against blood-borne antigens. However, its contribution to host immunity appear quite complex since there is convincing evidence

that antigen administered by other parental routes may pass through regional nodes and ultimately sequester in the spleen. Similarly, known patterns of lymphocyte traffic include the movement of antigen-reactive cells from stimulated nodes through the blood stream into the spleen where they lodge and initiate antibody secretion. Recent reports describing an apparent preferential localization of suppressor cells within the spleen certainly suggest that this organ may also play an important role in modulating established immune responses.

Studies outlined in our last annual report provided the first anatomical description of deep lymphatics with the rat spleen and demonstrated that this complex perivenular and periarteriolar network provided a major exit pathway for lymphocytes moving across both the red and white pulp. Over the past year these studies have extended to include serial, thin sections through the splenic hilus which revealed that true endothelial-lined lymphatic vessels passed along hilar vessels and penetrated the splenic capsule to merge directly with channels formed between concentric layers of reticular cells in the periarteriolar lymphatic sheath and provided a continuous conduit for egress of lymphocytes leaving the splenic white pulp. When combined with our previous autoradiographic studies on the sequential movement of radiolabeled lymphocytes through the spleen, this demonstration of anatomical continuity between true lymphatics in the hilus and "pseudolymphatic channels" in the white pulp should resolve much of the current conflict over the routes lymphocyte recirculation in the spleen. Further, study of these autoradiographic slides after prolonged exposure to permit differentiation between heavily and lightly labeled cells have now established similar traffic patterns for T and B cells within the spleen. Our studies indicate that both classes of lymphocytes move into the white pulp by entering the sheath directly through segments devoid of a defined marginal zone (the "bridging zones of Mitchell") or moving through intercellular gaps between macrophages in the marginal zone. Between one and six hours after intravenous infusion, radiolabeled T and B lymphocytes were randomly dispersed throughout all layers of the sheath. However, sections examined at eight to 24 hours post-infusion showed a definite sequestration of lightly labeled cells in the corona surrounding germinal centers. This finding and our observation that virtually all labeled lymphocytes entering hilar lymphatics between three and eight hours were heavily-labeled provided evidence for rapid T cell transit across the splenic white pulp which differed from the relatively slow movement of B cells which appeared to sequester about germinal centers. In terms of our current knowledge of cellular interactions in immunity, this traffic pattern appears to permit exposure of recirculating lymphocytes to antigens sequestered along the marginal zone or in the interstices of the sheath, followed by a random intermixing of T and B cells within the sheath which may facilitate cellular collaboration and then diversion of these cell populations into different anatomical regions of the white pulp.

With this traffic pattern established, our efforts have been directed at defining the mechanisms which provide the driving force for propelling lymphocytes through the splenic white pulp. Direct injection of dilute India ink into the splenic artery in anesthetized rats caused immediate, diffuse staining of all splenic lobules. Some carbon particles passed through the spleen and emerged in efferent veins within seconds after injection, but no staining was observed in hilar lymphatics. When these spleens were excised, sectioned, and examined by gross and light microscopy, there were no indications that this large, particulate tracer ever penetrated into the splenic white pulp, trabecular, or hilar lymphatics. Similarly, direct injection of India ink into the splenic parenchyma caused dense staining of the adjacent red pulp and prompt appearance of carbon particles in the hilar veins without any signs for entry of this tracer into splenic lymphatics. When the same experiments were repeated using patent blue dye (a low molecular weight tracer which has been widely used in defining peripheral lymphatic vessels), the injection of a small bolus of dye into the splenic artery caused immediate bluing of the entire spleen. Dye appeared in the spleen within seconds after injection, and after a delay of 2 - 3 minutes, a definite blue tinge was apparent in the hilar lymphatic vessels. Since this same sequence of events was reproduced in each of the 12 rats injected, it provided strong evidence for the recovery fluid transudates containing macromolecules by a deep lymphatic system within the spleen. Similarly, local injections with 0.05 ml. of patent blue into the substance of the spleen caused focal staining of the adjacent red pulp with immediate appearance of dye within hilar veins. After this initial bolus had been cleared, the veins refilled with undyed blood and resumed their normal appearance. At this time (40 - 80 seconds post-injection) the hilar lymphatics draining the injected bbule filled with clear blue fluid which gradually flowed through major extrasplenic lymphatic trunks to enter the thoracic duct. Despite obvious limitations imposed by needle tract trauma and elevated injection pressures, this flow of fluid containing low molecular weight tracers from venous sinusoids into lymphatics may mimic the normal physiologic process since only the blue dye appeared in hilar lymphatics when an additional 6 rat spleens were injected in the same manner with a mixture containing both India ink and patent blue dye. Further, direct visual examination of cut tissue sections from all spleens injected with patent blue dye revealed definite blue staining of both the red and white pulp. Since the tissue concentrations of the dye achieved by these in vivo staining techniques were too low to permit accurate tracing of the pattern of dye localization within the white pulp, similar studies were made using horseradish peroxidase (HRP) as the tracer material.

Following direct injection of a single bolus (0.05 - 0.1 ml.) of 0.05% HRP in saline into the splenic artery, rat spleens were excised and prepared for enzyme histochemistry using techniques described in our previous progress

reports. In tissue sections obtained at 10 seconds post-infusion, dense deposits of reaction product were observed in the lumens of both arteries and veins within the spleen. Staining of the red pulp varied from faint to intense in the splenic sinuses. In some areas a faint haze of tracer was seen extending from the red pulp across the marginal zone into the white pulp. At one minute post-injection, denser deposits of reaction product were found in the sinuses adjacent to the marginal zone and a gradient of decreasing staining intensity was seen extending from the marginal zone towards the central arteries of the white pulp. By two minutes, this gradient was no longer visible and reaction product appeared evenly distributed over the red and white pulp. At 3 minutes, exogenous peroxidase activity had virtually disappeared from the red pulp while the white pulp still retained scattered deposits of reaction product. After 5 minutes, the entire spleen was devoid of exogenous peroxidase activity and closely resembled non-perfused spleens where red pulp could be differentiated from white pulp only by reaction product deposition over red cells which filled the sinusoids. This correlation between the apparent flow of a bolus of HRP across the spleen seen in histologic sections and the transit of patent blue dye observed in intact rats suggested that transudates from the sinuses give rise to a fluid wave which sweeps macromolecules and cells across the red pulp into the white pulp and lymphatic vessels which exit at the splenic hilus.

In light of our present knowledge of splenic anatomy, these results can best be interpreted as follows. The direction of blood flow in the spleen is through major arteries entering at the hilus, out into white pulp arteries which terminate in the marginal zone and pulp cord. Blood then flows back into venous sinuses and trabecular veins which return to the splenic hilus. The long-standing controversy over whether this represents an open or closed circulation has been resolved by scanning electron microscopy which demonstrated that cordal spaces were partitioned by large reticular cell processes forming a rather complete channel conducting blood from the arterial ending to the intraendothelial slits in the sinus wall. While it is possible that this channel may be disrupted by physiologic processes which divert blood from the sinus walls and force fluid transudation, available evidence suggests that the lymphatic dependent fluid wave probably originates in the splenic sinuses due to contraction of a sphincteric mechanism at their efferent ends as described by Knisely. Sinus filling behind these contracted sphincters probably causes transudation with fluid flow back across the red pulp, through the marginal zone into the lymphatic sheath and ultimately into efferent splenic lymphatics leading to the thoracic duct. It is this fluid wave which probably provides the driving force for rapidly propelling lymphocytes across the spleen into periarterial and perivenular lymphatics described in our previous studies. Further evidence for this concept is presented in subsequent sections of this report which establish that lymphocytes rendered non-motile by treatment with Cytochalasin A continue to move through the spleen in a nearly normal manner.

PROJECT II. Patterns of Lymphocyte Recirculation in Normal Animals

Previous reports have concluded that large or immature lymphocytes isolated from the thoracic duct, spleen, lymph nodes, and thymus do not recirculate, but appear to leave the circulation at random to enter tissues subjected to intensive antigen exposure such as the gut. Griscelli's finding that large lymphocytes isolated from rat mesenteric nodes tended to preferentially accumulate in gut-associated lymphatic tissues, while cells from other nodes lodged preferentially in peripheral lymphatic tissues, have been attributed to differences in the distribution of antigen stimulation in normal animals. This finding has not been considered to be an important regulatory factor of lymphocyte traffic in intact animals where immature cells probably do not recirculate. Indeed, Strober described two different patterns of traffic for stimulated B lymphocytes. "Primed B-cells" isolated early in the immune response tended to lodge in the spleen after transfusion and did not recirculate while "memory cells" isolated in later stages after immunization had acquired the capacity to recirculate through peripheral lymphatic tissues and could be recovered from the thoracic duct. While there is strong evidence indicating that long-lived T and B cells recirculate at different rates through peripheral lymphatic tissues, most observers have postulated that these cells randomly distribute in the lymph nodes of normal animals. This tennant has been challenged recently by Cahill who has concluded that recirculating cells in thoracic duct lymph contain large numbers of lymphocytes which preferentially home into gut-associated lymphatic tissues, while the cellular efflux from popliteal nodes is composed primarily by lymphocytes which "home" into peripheral lymph nodes. Since this concept of subsets of recirculating lymphocytes which exhibit different traffic patterns could negate most of the established literature on lymphocyte recirculation and influence our studies on cellular traffic during immunopotentialiation, we have undertaken a series of experiments designed to provide the first, comprehensive quantitative description of the traffic of thoracic duct lymphocytes through all major lymphatic tissues of normal rats at varying ages of maturity.

To achieve this goal, we first autopsied groups of 25 normal female Lewis rats weighing 30 to 40 g., 80 to 90 g., and 190 to 200 grams and weighed their lymph nodes and spleens to estimate the total mass of organized lymphatic tissue. The lymph nodes were then subgrouped according to the origin of their lymphatic drainage to simplify comparisons of potential regional differences in cellular traffic as follows: Group AB consisting of the axillary, brachial, inguinal, and popliteal nodes which drain the skin of the flank and extremities; Group SM which included all submandibular and cervical nodes draining mucous membranes of the mouth and oropharynx; Group MES consisting of all nodes in the mesenteric lymphatic chain; Group PP composed by the 16 to 22 discrete Peyer's Patches

scattered along the wall of the small intestine and Group OTH consisting of a heterogeneous group of nodes draining the lung, peritoneum, and urogenital system. The results shown in Table I show that there is a progressive increase in both lymph node and splenic mass as weanling rats mature into adulthood. The total adult lymph nodal weight of some 700 to 750 mg. is achieved when femal rats are about half-grown and then maintained at about this level throughout the first year of life. It is equally interesting that the gut-associated lymphatic tissues (mesenteric nodes and Peyer's Patches) account for nearly half the total lymphatic tissue mass at each developmental stage studied. On the basis of these findings alone, it seems possible that previous descriptions of the apparent preferential homing of thoracic duct lymphocytes into gut-associated lymphatic tissues which were made without considering the total lymph nodal mass might simply reflect the relatively large size of the mesenteric lymph nodes.

This question was analyzed by following the tissue distribution of radioactivity in 32 rats from each developmental stage at sequential time intervals after infusing 300×10^6 H-uridine labeled, syngeneic thoracic duct lymphocytes. The results of these studies are shown in Table II to IV. In adult animals weighing 190 to 200 g., the bulk of the transfused lymphocytes were transiently held up within the vasculature of the lungs, liver, and spleen over the first half hour post-injection. Then, these organs rapidly lost radioactivity as labeled cells accumulated in the spleen over the next one to two hours. Thereafter, the total counts within the spleen and other viscera progressively declined while radioactivity continued to rise within lymph nodes until a peak level was reached at 18 to 24 hours. Since comparable infusions with heat-killed labeled lymphocytes or free ^3H -uridine alone failed to yield significant accumulation of radiolabel within either the spleen or lymph nodes, this pattern of redistribution of radiolabel within the body clearly resulted from the traffic of viable lymphocytes into lymphatic tissues. Similar patterns of sequestration and redistribution of radioactivity were observed in half-grown rats weighing 80 to 90 g. (Table III). Since rats at this age have a relatively small body mass and lower numbers of recirculating lymphocytes to populate the organized lymphatic tissues which have developed into adult size, it might be anticipated that the standard inoculum of 300×10^6 cells used in these studies should produce an early and more rapid movement of radiolabel into lymph nodes. This was, in fact, observed in all animals. However, it should also be recognized, in light of our splenectomy studies discussed in subsequent sections of this report, that smaller spleen size and concomitant reduced early accumulation of radiolabel within the spleen may indicate that this organ sequesters fewer recirculating lymphocytes than adult spleens and thus permits greater uptake of infused cells within peripheral lymphatic tissues. The observations of radiolabel cell traffic in 30 g. rats are of equal interest. Since these animals were found to have a total lymph node and splenic mass less than half that present in mature

adults, rapid expansion of their relatively small pool of recirculating lymphocytes by the standard inoculum of 300×10^6 thoracic duct cells might be expected to cause a similar early increase in the lymphatic tissue uptake of radiolabel. However, data presented in Table IV demonstrate that both the rate and total accumulation of labeled cells within their lymphatic tissues was significantly less than that seen in adult rats. These findings certainly suggest that nodal blood flow, the total surface of HEV available for emigration, or both factors must be so small in these young rats that even relative saturation of the available sites for emigration cannot achieve total organ accumulations of radioactivity comparable to that found in adult animals. While we do not yet have data available from our autoradiographic studies on the nodes of young animals, we have completed other experiments which support such conclusions. The mesenteric lymph nodes of developing Lewis rats ranging from 1 to 28 days of age were studied by both alcian blue perfusion, histochemistry, and routine light microscopy using methods described in our previous progress reports. The results showed that in the first 24 hours after birth, mesenteric lymph nodes were formed by primitive mesenchyme which was devoid of HEV and small lymphocytes. At three days, numerous small lymphocytes were seen in the primitive cortex of these nodes and this was accompanied by the appearance of high endothelial cells in a few small venules. At one week, the first medullary sinuses were seen in these developing nodes, but there was little medullary tissue so that the total node was dominated by a relatively thick cortex populated with lymphocytes. The HEV were present as short, vertically oriented vessels measuring some 200 to 400 μ g in length and they lacked the typical arborizing structure seen with maturity. Between 14 to 21 days, these nodes developed a better defined sinus system and enlarged, but they did not develop the typical, long branching network of HEV's we have described in mature rats until after four weeks of age. Further, attempts to estimate lymphocyte traffic into the two to three week old mesenteric nodes showed that the ratio of migrating lymphocytes/high endothelial cells was .52 - a value well below the .81 value observed in normal rats. Together, these morphologic demonstrations of decreased lymphocyte traffic across the short HEV segments of the developing node appear to be entirely consistent with our radiokinetic studies.

The decline in total recoverable radioactivity within the tissues studied in these experiments may be due, in part, to loss of recirculating lymphocytes through the intestine since 5 to 10% of the total dose were found with large and small bowel at 8 to 24 hours. However, the specific activity (DPM/g. of tissue) of the intestine was quite low when compared with that found in Peyer's Patches or peripheral lymph nodes, and our autoradiographic studies failed to demonstrate more than a very few labeled lymphocytes in the gut wall. In view of these findings, it seems more likely that the decline in recovered radioactivity may result from loss of ^3H -uridine from the labeled cells due to RNA turnover as our autoradiographic

studies showed clear evidence for a decline in grain counts within individual lymphocytes between 4 to 24 hours after infusion. Despite this limitation, the sequential shift in remaining radioactivity from the spleen into lymph nodes observed in all rats suggested that viable lymphocytes which had been sequestered initially within the lungs, liver and spleen were redistributing in peripheral lymphatic tissues. More direct evidence supporting the concept that this redistribution of radiolabel resulted from continual lymphocyte recirculation through peripheral lymphatic tissues was obtained by repeating these experiments in 200 gram rats bearing established thoracic duct fistulae. Results shown in Table V demonstrate that only small amounts of radioactivity appeared in thoracic duct lymph within the first two to four hours post-infusion, and this correlates well with our autoradiographic data which indicated that only a few labeled lymphocytes moved across peripheral lymph nodes to enter efferent lymph within two hours after transfusion. Yet, the transit times for the bulk of the infused cells must be considerably slower since less than 1% of the total injected radioactivity was recovered in thoracic duct lymph during the first four to eight hours while the total accumulation of radioactivity in the lymphatic tissues of the cannulated rats over the same time span was roughly comparable to that seen in normal animals. When lymph drainage was continued out to 24 hours, some 12 to 28% of the total injected dose could be recovered in thoracic duct lymph and this was paralleled by a significant reduction in the expected progressive accumulation of radioactivity within lymphatic tissues.

The observations described above provide strong evidence for the continued recirculation of thoracic duct lymphocytes with a traffic pattern characterized by early sequestration in the spleen which is followed by progressive redistribution of these cells in peripheral lymph nodes. Since results described in our last annual progress report described different pathways and transit times for lymphocyte movement across the red and white pulp, this early sequestration of radioactivity in the spleen probably reflects early accumulation of blood borne lymphocytes within the splenic sinusoids and it is not surprising that such cells would ultimately move into peripheral lymph nodes. However, Katz's group has described a lymphocyte sub-population which purportedly has the ability to selectively "home" into the spleen and fails to move into other organized lymphatic tissue. To determine whether thoracic duct lymph contained a similar population of "spleen-seeking" lymphocytes, we repeated these experiments in a series of adult rats which had been splenectomized seven to ten days prior to use for celltraffic studies. The results shown in Table VI demonstrated that there is an early and more rapid accumulation of radiolabeled lymphocytes in the peripheral nodes of splenectomized rats, but this rapidly plateaus so that the total radioactivity present in their lymph nodes at 8 to 24 hours is virtually identical to the combined total seen in the spleen and lymph nodes of normal rats. These findings mitigate strongly against the presence of a significant population of "spleen-seeking" lymphocytes in thoracic duct lymph and suggest that the initial differences in distribution of recirculating lymphocytes in various lymphatic organs are determined by differences in blood flow and tissue transit times.

If this thesis is correct, it might be anticipated that simply enlarging the splenic mass or disrupting its normal architecture could result in delayed transit and prolonged sequestration of recirculating lymphocytes within this organ. Since such mechanisms might contribute to the immune alterations seen in some infectious diseases, or the lymphopenia associated with splenomegaly, we have begun a series of experiments designed to provide new insight into this problem. Data presented in Table VII shows that splenic weight had increased by nearly 50% at one week after three daily IV injections with 10^8 sheep red blood cells while the mass of the peripheral lymph nodes was unaltered. Despite this evidence for induced splenomegaly which is known to be related to immune response, the splenic uptake and subsequent redistribution of labeled thoracic duct lymphocytes into peripheral nodes was unaltered in these animals. Similarly, normal traffic patterns were observed in rats bearing a syngeneic, transplantable lymphoma where our morphologic studies indicated that malignant lymphocytes have invaded both the splenic red and white pulp, expanding the total mass of this organ two to four times that seen in normal rats (Table VIII). These findings certainly demonstrate that there is no direct correlation between splenic size and the sequestration of lymphocytes from the recirculating pool and further studies are needed to define the precise mechanisms which regulate cellular traffic across the splenic parenchyma during immune responses and different disease states.

Having established a rather consistent pattern for exchange of recirculating lymphocytes between spleen and peripheral lymph nodes, additional studies were made to determine whether thoracic duct lymph contained significant numbers of cells exhibiting preferential "homing" into the gut-associated lymphatic tissue as postulated by Cahill. Results of studies showing the pattern of accumulation of radioactivity in different anatomical groups of lymph nodes in normal rats after infusing ^3H -uridine-labeled lymphocytes are shown in Table IX. These experiments demonstrate that when various groups of lymph nodes are examined without considering their relative size and different morphology, the total accumulation of radioactivity within the mesenteric nodes is consistently greater than that present in any other lymph node at each time interval studied. Conversely, Peyer's Patches display an early, rapid increase and gradual decline in radioactivity which is quite consistent with our previous observations that these specialized lymphoid structures lack medullary cords and may be exempt from the altered patterns of egress described following stimulation of peripheral nodes. Since each of the other lymph nodes groups which were roughly of comparable size, the greater number of total counts within the gut-associated lymphatic tissue might be attributable to the greater mass of the mesenteric nodes.

To clarify this issue, the same nodes were compared using specific activity (DPM/mg. of tissue) (Table IX). With this technique, the mesenteric nodes continued to show the greatest activity at each time interval, but after eight hours its specific activity was only slightly greater than that seen in the submandibular nodes. The Peyer's Patches showed the same biphasic rapid rise and decline in specific activity expected from the total organ counts. This discrepancy between the patterns of accumulation of radiolabeled cells within the major constituents of the gut-associated lymphatic tissues does little to support Cahill's concept of subpopulations with selective homing capacities. Indeed, our finding that the submandibular nodes contain nearly the same specific activity as mesenteric nodes suggests that the factors causing increased accumulation of recirculating cells in selected nodes may be related to changes induced by the intense background antigenic stimulation one would expect to exist in lymphatic tissues draining the oral and intestinal cavities. More direct evidence bearing on this problem was sought using radiokinetic analysis of cellular traffic in animals where the gut-associated lymphatic tissues were ablated selectively. In our initial trials the spleen, mesenteric nodes, and entire small bowel were excised to remove all gut lymphatic tissues and the remaining duodenal stump was anastomosed to the transverse colon. These animals were infused with the standard inoculum of 300×10^6 uridine-labeled TDL cells to determine what fraction of infused lymphocytes could localize in the remaining nodes. The results shown in Table X show that the initial accumulation of radioactivity within peripheral nodes of these rats was about half that seen in animals subjected only to splenectomy. (Table VI). However, from eight to twenty-four hours, the total radioactivity present within the remaining lymphatic tissue was only a small fraction of that seen in both normal and splenectomized rats. This dramatic fall in lymphocyte entry cannot be attributed to removal of the "homing" site for gut-seeking lymphocytes. Indeed, the extensive surgery, postoperative ileus, and profound malnutrition induced by the surgical manipulations almost-certainly evoked increased steroid secretion in response to stress, and there is ample evidence from other laboratories indicating that modest increases in the plasma cortisol level can block lymphocyte recirculation. To resolve this issue, identical traffic studies were repeated in rats where only the mesenteric nodes had been excised. These animals all tolerated the operative procedure well, maintained normal body weight, and appeared to rapidly re-establish lymphatic flow from the mesentery into the thoracic duct since no evidence for lymphatic fistulae or chylous ascites was found at autopsy. Table II shows the pattern of distribution of radioactivity in these animals when labeled lymphocytes were infused two weeks after surgery. Comparisons of this data and that presented in Tables II and IX for normal rats demonstrates that the total nodal uptake and sequential distribution of radiolabel was identical in both groups. This finding and the absence of any increased accumulation of label with the Peyer's Patches virtually excludes the presence of significant subpopulations of gut-seeking lymphocytes within rat thoracic duct lymph. Thus, it seems likely

that variations in regional blood flow and microenvironmental changes induced by antigen challenge may be the major factors determining the distribution of recirculating lymphocytes within different lymph nodes and this documentation provides a reasonable basis for our future attempts to promote immunopotentiality by selectively manipulating this cell traffic.

To provide more detailed information on cellular traffic in normal rat lymphatic tissues, we have initiated detailed autoradiographic studies to define the rate of entry, intranodal distribution and transit times of labeled lymphocytes in Peyer's Patches, mesenteric, axillary and submandibular lymph nodes. The results obtained to date show a remarkable correlation with the whole organ kinetic data with the added dimensions of being able to follow and localize the traffic of both T and B lymphocyte subpopulations in these nodes. Table VI shows the quantitative data compiled by Major Anderson localizing labeled lymphocytes within various anatomical subdivisions of the lymph nodes at sequential intervals post-infusion. These findings clearly establish that lymphocytes can move from the blood, across the HEV wall into the parenchyma of all node types within three minutes after intravenous injection. This can be explained only by rapid cellular transit across the walls and surrounding sheaths and there does not appear to be any significant differences in this sequence within different nodes. Thereafter, a progressive increase in the total number of labeled cells is seen within the diffuse cortex with time and this increase shows a rank order of mesenteric nodes - submandibular nodes - axillary nodes similar to that noted in the whole organ studies. These differences cannot be attributed to impaired egress since labeled cells move into efferent sinuses within 30 minutes in all nodes and the total number of exiting lymphocytes is actually considerably greater in the mesenteric nodes between one-half to two hours. Since these histologic scoring techniques do not consider total nodal mass, the best explanation for the progressive increase in labeled cells within the mesenteric nodes may be increased blood flow to this gland or selective homing as described previously. These findings also support our thoracic duct fistulae studies where small quantities of label were recovered in efferent lymph within two hours, but the bulk of the injected cells did not enter thoracic lymph until after six hours. We have not yet completed studies of the Peyer's Patches, but the finding of numerous labeled lymphocytes within the cortical sinuses at one hour post-injection certainly suggest that even more rapid cellular transit may occur in these specialized lymphatic tissues. When these autoradiographic sections were reviewed using dense and light labeling to differentiate between recirculating T and B cells, other interesting aspects of this cellular traffic are becoming apparent. The data indicates that both T and B lymphocytes emigrate across HEV walls at similar rates, but there is a suggestion that B cells may preferentially leave the blood by moving across distal segments of the HEV. In the early intervals, both lymphocyte classes appear to be randomly

dispersed in the diffuse cortex and we have not been able to document the reported segregation of these cells into T and B zones as yet. Despite this, it is already apparent that the bulk of the lymphocytes moving into the medullary sinuses and efferent lymphatics of the mesenteric node in the first two hours are heavily-labeled T cells so that the diffuse cortex contains a relative increase in B cells. This provides strong evidence for rapid T cell transit across the mesenteric node while B cells appear to move more slowly across this node causing an apparent retention of B cells which really reflects total lymphocyte traffic across the cortex. We are now expanding these studies to later time intervals and beginning similar experiments in adjuvant-treated rats to attempt better definition of the mechanism responsible for differences in cellular traffic in both normal and altered nodes.

PROJECT III. Studies on the Mechanisms of Lymphocyte Entry into Lymph Nodes.

Morphologic evidence summarized in our previous annual progress reports has provided a firm base for the concept that lymphocyte entry into lymph nodes depends upon the selective attachment of blood-borne lymphocytes to the surface of high endothelial cells followed by active migration of these cells across the venular wall. The precise biochemical mechanisms regulating this process are still controversial. Postulates that attachment depends upon surface recognition between complementary receptors on lymphocyte and endothelial cell surfaces are based largely upon enzymatic digestion studies showing that brief treatment with dilute trypsin and a few other selected enzymes can both prevent lymphocyte recirculation and dislodge adherent lymphocytes from the luminal surfaces of HEV. Other methodologies are clearly needed to better define this critical step in regulating lymphocyte recirculation. Since defined lymphocyte populations can be readily obtained for experimental use, the major roadblock for definitive studies in this area has been the complete lack of suitable means for isolating endothelial cells. Over the past year we have attempted isolation of HEV endothelium using collagenase, pronase, hyaluronidase, trypsin, and EDTA alone or in varying combinations with minced lymph nodes. None of these approaches have provided adequate isolation of HEV endothelial cells. Related efforts attempting to dislodge these endothelial cells by enzymatic perfusion of the isolated lymph node vasculature have also failed. Since these approaches have been unrewarding thus far, we have evaluated an alternate approach described by Woodruff. In brief, her method consists of incubating a suspension of freshly isolated lymphocytes over cryostat sections of lymph nodes using gentle agitation on a mechanical shaker to distribute lymphocytes over the tissue section. The specimens are then washed and fixed in 3% glutaraldehyde and stained with methyl-green pyronin. With this approach, Woodruff has claimed that some 64 to 80% of the cells remaining adherent to the tissue sections adhere selectively to HEV surfaces. We have investigated

this approach carefully by: (1) using both thoracic duct and peripheral blood lymphocytes collected in such anticoagulants as heparin, citrate, EDTA; (2) using fresh unfixed cryostat sections with omission of the fixation and staining procedures and scoring the specimens by phase microscopy; (3) using a variety of different buffers to maintain pH in the 7 to 7.4 range throughout the entire procedure; (4) omitting one or both steps of glutaraldehyde fixation described by Woodruff; (5) substituting several different stains for the methyl green pyronin used in the reported technique. In brief summary, neither the basic technique reported by Woodruff, nor any of the various modifications we have introduced, have provided a convincing demonstration for selective attachment of the suspended lymphocytes to HEV surfaces. We have observed that many lymphocytes do adhere to the tissue section, particularly when the double glutaraldehyde fixation steps employed by Woodruff are utilized. However, this binding appears to be entirely non-specific for when the sections are scored by a neutral observer as many or more lymphocytes are found adherent to walls of medullary sinuses and cracks or knife cuts in the section than are present over HEV's. We have communicated these disturbing results to Dr. Woodruff, and she admits to having some difficulties in reproducing her original data in her own laboratory; but she also believes that these observations are correct and has invited us to visit her laboratories for mutual exchange of information on this technique. Further evaluation of this technique is planned in the near future, but until convincing, reproducible demonstrations of selective lymphocyte attachment has been achieved with this model it should be recognized that double fixation with glutaraldehyde utilized by Woodruff may simply reflect artifacts induced by cross linking between lymphocyte surface proteins and globulins within the tissue section.

This inability to accurately define the surface recognition mechanisms responsible for cellular traffic in lymph nodes has compounded the current controversy over the existence of lymphocyte subpopulations which purportedly "home" selectively into specific lymphatic tissues - i.e. the spleen-seeking and GALT-seeking lymphocytes discussed in Project II. When these considerations are extended to include lymphocyte populations other than those obtained from lymph or blood, this confusion becomes more pronounced. Other investigators have attempted radio-kinetic measurement of cellular traffic using heterogeneous lymphocyte suspensions obtained by mechanical or enzymatic disruption of the spleen, lymph nodes, Peyer's patches, bone marrow, and thymus. While such reports have concluded that large lymphoblasts which do not normally recirculate can lodge in the lamina propria of the gut wall or sequester in or near germinal centers within the spleen and lymph nodes, it has been difficult to estimate the extent of this traffic since these preparations contain large numbers of injured and dead cells which cause marked accumulation of radiolabel within the liver, lung, and gut. Despite these limitations, such findings certainly suggest that most lymphocyte subpopulations possess

the capacity to emigrate into established lymphatic tissue so this "homing" instinct cannot be entirely dependent upon cellular maturation or their ability to recirculate. This sort of reasoning might well lead to the conclusion that surface recognition mechanisms are characteristic of all lymphoid cell lines except for those who lose this capacity to re-enter lymphatic tissue when they differentiate and mature into short-lived effector cells. If this thesis is correct, it seems plausible that the different patterns of tissue and organ involvement seen between lymphomas and lymphatic leukemias may be determined by the presence or absence of surface receptors which regulate lymphocyte recirculation. Lymphoma cells may possess the receptors required for "homing" into lymph nodes, while similar recognition mechanisms may be lacking in leukemic cells. This would permit both types of malignant lymphocytes to spread within the sinusoidal circulation of the marrow, liver, and spleen; yet, only lymphoma cells would preferentially localize in peripheral nodes. The late appearance of a leukemic phase in some lymphomas could simply reflect the saturation of nodal tissues, destruction of high endothelial venules, or loss of surface receptors due to genetic drift.

We have begun to test this hypothesis using a B-cell lymphoma produced in Lewis rats which is readily transferred in four to six week old syngeneic recipients. Subcutaneous inoculations with 10^3 to 10^6 spleen cell suspensions or isolated peripheral blood cells from tumor bearing hosts result in local nodular growth within five days which is followed by blood invasion with hematogenous spread of tumor cells to the spleen, liver, and lungs by eight to ten days and then the rats die in a leukemic phase at 14 to 21 days post-inoculation. In the late stages, peripheral blood counts range from 2×10^5 to 2×10^6 cells/mm³ and some 99% of these cells are pleomorphic, blastoid cells when examined using Wright's stain. Lymph node invasion with these malignant lymphocytes is not seen in any nodal group other than that draining the local tumor and thoracic duct lymph isolated from these rats contains approximately the same # and morphology of lymphocytes seen in normal, non-tumor bearing rats. These findings certainly suggest that this experimental lymphoma lacks the surface receptors required for entry into peripheral lymph nodes. Further, when malignant lymphocytes are obtained from peripheral blood from tumor bearing rats, a 100% viable population of malignant lymphocytes is obtained which avidly incorporate ³H-uridine during brief, in vitro incubation. Lymphocyte traffic data from such preparations is shown in Table XIII, and this certainly establishes that these malignant lymphocytes fail to accumulate in peripheral nodes in spite of their propensity for producing massive blood invasion. Since this tumor line provides the first definitive proof for a non-effector lymphocyte population which cannot "home" into lymphatic tissues, further experiments along this line and comparisons with normal lymphocyte populations may significantly enhance our understanding of the mechanisms regulating lymphocyte recirculation.

Scanning and transmission electron microscopic studies described in our previous annual progress reports have strongly suggested that motile lymphocytes actively migrate across HEV walls as they leave the blood stream and enter the nodal parenchyma. We have now completed a series of experiments designed to test this postulate in a more direct manner. Initially, thoracic duct lymphocytes were incubated in vitro with Cytochalasin A at concentrations ranging from 5×10^{-3} to 5×10^{-8} M for 30 minutes. Then, the cells were washed, resuspended in M-199, and examined for motility using both the heated microscopic stage and the migration under agarose assay (see Project IV). These studies demonstrated that Cytochalasin A at concentrations of 5×10^{-5} to 1×10^{-4} M completely inhibited random lymphocyte movement in both assays. Further, this inhibition appeared to be irreversible under the conditions used in vitro since no random movement was found when washed cells were incubated at 37° C under agarose for 12 to 24 hours. This suppression of cell motility could not be attributed to cell death since trypan blue dye exclusion studies indicated that the cell preparations maintained 95% viability through this 12 to 24 hours period. Examination of these Cytochalasin-treated cells by both scanning and transmission electron microscopy in collaboration with Doctor John White and Major Arthur Anderson at USAMRIID showed that these lymphocytes exhibited a marked decrease in the number of microvillous projections with frequent clustering of the microvilli at one pole of the cell with their distortion into elongate, club-shaped or bleb-like forms. Transmission EM documented that these surface changes were caused by disruption and aggregation of microfilaments within these cells which were identical to reported descriptions of the Cytochalasin-induced alterations described in other cell types.

Having proven that in vitro Cytochalasin treatment had inhibited lymphocyte motility without causing overt cellular injury, we then repeated our in vivo traffic studies following the sequential organ distribution using this lymphocyte population. The results shown in Table XIV prove that the accumulation of lymphocytes within peripheral lymph nodes is almost completely blocked by prior treatment with Cytochalasin. This cannot be attributed to non-specific sequestration of these cells within other capillary beds, since total uptake and redistribution of radiolabel within the lungs and liver is virtually identical to that seen using normal TDL cells (See Project II). Further, the striking differences seen between the distribution of Cytochalasin-treated cells and control inoculae using heat-killed lymphocytes mitigate against cell death as being a significant contributor to this altered recirculation pattern. It is equally interesting that while the entry of Cytochalasin-treated lymphocytes into peripheral nodes is markedly depressed, a significant proportion of these cells continued to redistribute in the spleen in a manner analogous, but somewhat reduced, to that seen with normal lymphocytes.

Other experimental methods were employed to confirm and expand conclusions derived from the radiokinetic studies. When 3×10^8 Cytochalasin-treated lymphocytes were infused into normal rats, histologic examination of their lymph nodes at sequential intervals post-transfusion showed a striking, sustained increase in the number of lymphocytes present within the lumens and walls of HEV than was produced by comparable infusions with normal TDL cells (Table XV). Analysis of these events by autoradiographic techniques demonstrated that this increase resulted from the accumulation of Cytochalasin-treated cells along the luminal surfaces of HEV and the concomitant failure or marked delay of these lymphocytes to migrate across the HEV wall into the adjacent nodal parenchyma (Table XVI and XVII). This clearly differed from the rapid movement of normal labeled lymphocytes through the venular wall which was found in all control animals within three minutes after the infusions were completed. The Cytochalasin-treated cells continued to accumulate within HEV walls over the initial two hours and then a few cells appeared to move into the surrounding sheath and assume a perivenular distribution. However, significant numbers of these cells were never found in the nodal parenchyma or efferent lymphatic channels, even when observations were extended to eight to 12 hours. When combined with the *in vitro* and cell traffic studies described previously, these results provide convincing evidence that lymphocytes can both recognize and attach to endothelial surfaces despite disruption of microfilament function which blocks their motility. Thus, our morphologic evidence indicating that active cellular motility is required for lymphocyte entry into peripheral nodes seems well substantiated. At the same time, it should be recognized that our present data showing near-normal accumulation of radiolabel within the spleens of these animals, is entirely consistent with the autoradiographic studies of Cytochalasin-treated lymphocytes within the spleen described in our last annual progress report. When combined with our evidence for a fluid wave in the spleen, these composite findings indicate that fluid flow - not cell motility - may be the main force propelling lymphocytes through the loose reticular meshwork of the spleen.

PROJECT IV. Studies on Lymphocyte Chemotaxis: Its Role in Lymphocyte Recirculation and In Vitro Assays of this Phenomenon.

Ultrastructural observations described in our previous annual progress reports have shown that when lymphocytes move across the HEV wall, they actively migrate through intercellular spaces which are contiguous with the venular lumen and the nodal interstitium. Since we have demonstrated that macromolecular tracers can pass from the lymph node into venous blood and surround emigrating lymphocytes in the process, we have postulated that lymphocyte movement across the HEV wall and surrounding sheath may be

directed by migration along a chemotactic gradient. While this is an appealing postulate, it has not yet been confirmed by more definitive experiments. Because there is still intense controversy over the natural mediators of leukotaxis in vivo and virtually nothing is known about specific lymphocyte chemotaxis, we have begun a series of experiments attempting to resolve some of these issues.

Our initial efforts have been directed at defining the effects of the microtubular disrupting drug- Colchicine - on lymphocyte migration using both in vitro and in vivo assays. This pharmacologic approach has obvious weaknesses, since Colchicine can ablate microtubular dependent secretion in all body tissues and produce a variety of toxic side effects which complicate interpretation of the experimental findings. However, the following data does provide evidence suggesting that lymphocyte entry into lymph node may be dependent upon secretory processes which are disrupted by Colchicine treatment. In vitro studies showed that rat thoracic duct lymphocytes incubated in tissue culture media containing Colchicine at concentrations ranging from 10^{-8} to 10^{-2} M for intervals ranging from one to 24 hours remained viable by trypan blue dye exclusion unless the Colchicine level exceeded 10^{-3} M. The effects of similar concentrations of Colchicine on lymphocyte motility were assessed by examining aliquots of these suspensions at phase microscopy and measuring random lymphocyte migration under agarose. Both techniques documented that lymphocytes retained normal motility until the toxic level of 10^{-3} M was achieved. Examination of Colchicine-treated cells (0.5×10^{-5} M) by scanning and transmission electron microscopy revealed that virtually all lymphocytes retained their normal surface conformation with numerous microvillous projections. While we were unable to demonstrate aggregated or dissociated microtubules in these cells, this may simply reflect the difficulty in documenting such changes within cells which lack a prominent microtubular network. Certainly, the inhibition of microtubular assembly has been substantiated in a variety of other cell types treated with comparable dosages of this drug and has been widely accepted as a general biologic response to Colchicine.

When ^3H -uridine labeled thoracic duct cells were incubated with 0.5×10^{-5} M Colchicine, washed, and transfused into normal rats, they exhibited the same pattern of uptake and redistribution within body organs as that seen using normal cells (Table XVIII). Further, when the lymphatic tissues from these rats were examined by autoradiography, the radiolabeled cells appeared to attach to HEV surfaces, emigrate into peripheral nodes, and move across the nodal interstitium in a completely normal manner. Reciprocal experiments were then made by infusing normal ^3H -uridine labeled lymphocytes into rats injected IP with Colchicine (0.2 mg./100 gram body weight) one hour before transfusion. Examination of the whole organ accumulation of radioactivity in these rats at sequential intervals

shown in Table XIX indicates that Colchicine-treatment markedly suppressed lymphocyte movement into lymph nodes and spleen for eight to ten hours without causing toxic sequestration of the label within the RE system of the liver and spleen. This effect appeared partially reversible in that progressive accumulation of the label was observed between eight to 24 hours. However, this was probably due to recovery from the inhibitory changes induced by a single bolus of Colchicine since further accumulation of radiolabeled cells within lymphatic tissues was prevented by administering a second injection of Colchicine at eight to ten hours. When combined with results described in our last annual progress report which showed that Colchicine caused marked reduction in the lymphocyte migration index, cellular depletion in lymphatic tissues and a progressive fall in thoracic duct lymphocyte output, the present experiments provide further proof that this drug blocks lymphocyte entry into lymph nodes. Since our in vitro studies and reports by others have established that this effect cannot be attributed to altered lymphocyte motility, we have attempted further definition of the possible mechanisms by ultrastructural studies on the nodes from Colchicine-treated rats. These observations have documented a marked decrease in the number of emigrating lymphocytes within the lumens and walls of HEV within one-half to one hour after Colchicine injection which is sustained for 12 to 18 hours. Despite this decline in number, the few migrating lymphocytes remaining at this site exhibit normal morphology, cytoplasmic organelle distribution, and intact microtubules. Thus, there were no indications that Colchicine treatment of the intact animal caused any structural alterations in recirculating lymphocytes which differed from those noted in vitro. The paucity of emigrating lymphocytes made it difficult to assess cytoplasmic polarity indicative of directional movement in a truly statistically significant manner, but we have been able to show definite evidence for continued directional movement across the HEV wall at time intervals ranging from one-half to eight hours post-Colchicine treatment. This finding might be interpreted as indicating that secretion of the putative chemotactic agent within the node is not totally suppressed by the dose of drug used, despite ultrastructural evidence for description of the labile microtubular network in high endothelial cells, macrophages, and large lymphoblastic cells. However, our studies have clearly indicated that lymphocyte attachment to HEV surfaces is almost totally absent within lymph nodes after Colchicine treatment. Since microtubules appear to be of great importance in the maintenance of the cytoskeleton and topographic stability of the membrane in addition to their contribution to intracellular organelle movement during secretion, it is also possible that Colchicine treatment has altered the membrane receptors on HEV endothelial cells in some manner which precludes lymphocyte attachment. Further experiments are needed to document the precise mechanisms responsible for blockade of lymphocyte recirculation by this drug.

Chemotaxis has been defined as "a reaction by which the direction of locomotion of cells is determined by substances in their environment. If the direction of movement is not definitely toward or away from the substance in question, chemotaxis is indifferent or absent" (White, 1974). Thus, only

directional migration can be used as a measure for chemotaxis, yet much of the literature on cellular locomotion has failed to establish this critical point and inadequate techniques have frequently been employed to measure this phenomenon in a confusing and misleading way.

The controversy over directed leukocyte locomotion is most pronounced over lymphocytes. Lymphocyte motility was beautifully documented by Lewis in the 1930's, yet numerous attempts to demonstrate a true chemotactic response for these cells failed and in 1974, White concluded that "the literature is singularly bereft of findings supporting the existence of chemotactic factors for lymphocytes." Ward's group first reported evidence that supernatants from antigen-stimulated lymph node cultures were chemotactic for all types of leukocytes - including lymphocytes - while using a Boyden chamber assay in 1971. This report has never been confirmed, but in a 1976 workshop at NIH, Ward indicated that he now had further evidence for lymphocyte chemotactants in supernatants obtained from Concanavalin-A stimulated lymphocytes, mixed leukocyte cultures, and anti-immunoglobulins. Regrettably, he has never indicated whether these agents attract all types of leukocytes as originally reported, or if these were truly specific in producing selective lymphocyte chemotaxis. With this possible exception, the only other documentation of selective lymphocyte chemotaxis has been reported by Higuchi, et al., as a fragment of 14000 MW cleaved from rabbit IgM by an SH-dependent protease isolated from granulocytes, and this response was also measured using a modified Boyden chamber.

Since lymphocytes are actively motile, it has long been suspected that the difficulties encountered in studying lymphocyte chemotaxis reflected limitations of the existing in vitro assay procedures. While Cebra's criticism that it is impossible to differentiate between random and directional movement in the two chamber system employed by Boyden may not be entirely correct, this technique certainly has many inherent limitations which make it difficult to achieve adequate quantitation and reproducibility - i.e. (1) maintaining concentration gradients over a time interval that allows cells to migrate appreciable distances indicative of a directional response; (2) providing adequate morphologic identification of the responding cells; (3) variable ability of different cell populations to adhere to cellulose filters to permit migration and frequent detachment of these cells from the underside of the filters; (4) widespread use of dilute serum, anti-coagulants, or gelatin in the suspensions which may alter responses to possible chemoattractants; etc.

These criticisms of the Boyden chamber and its multiple modifications have lead us to attempt development of another methodology for studying lymphocyte chemotaxis in vitro. In the past year we have concentrated our efforts on

modifying the Nelson method for studying cellular migration under agarose since this provides a three chamber technique for simultaneous comparison of both directional and random migration over relatively long time periods. After numerous trials, we have succeeded in developing this assay method to the state where it appears to negate many of the deficiencies associated with the Boyden chamber. While Nelson has never observed either random or directional migration of lymphocytes in his assay, our modifications of this technique have provided clear cut and reproducible demonstrations of both types of lymphocyte movement in vitro.

In our modification, sterile MEM containing 2 m M glutamine, 375 g/ml NaHCO_3 , and 69 m M Hepes buffer was added to 0.8% agarose dissolved in Hepes buffer. This mixture was mixed with a mechanical stirrer and poured into sterile Petri dishes where six circular coverslips were positioned on its bottom in a hexagonal array. After gelation, a template was used to cut three wells, 2.44 mm. in diameter, and separated by 2.4 mm. over each coverslip. After aspirating the agarose plugs, the plates were pre-incubated for three hours under 5% CO_2 and saturated H_2O atmosphere at 37°C . The washed cell suspensions (thoracic duct lymphocytes, peripheral blood leukocytes obtained using Ficoll-Hypaque separation, peritoneal macrophages, spleen cells, etc.) were added to the center well over each cover slip while putative chemotactic agents and control solutions were placed into the outer wells. The plates were incubated under 5% CO_2 saturated H_2O atmosphere at 37°C , and cellular migration under agarose adjacent to the center well was scored using an inverted phase microscope. At selected intervals, ranging from one-half to 24 hours, some plates were fixed, dehydrated, and separated from the gel as described by Nelson. The coverslips were removed, stained with Gremia, and mounted on glass slides to provide permanent specimens. These were examined by light microscopy to establish the morphology of the motile cells and an ocular micrometer grid system was used to measure the distance of cellular migration from the edge of the center well. Standard stem and leaf plots were used to illustrate the number of migrating cells and the distances they had migrated under agarose along a line connecting the centers of all three wells. Measurement of the linear distance covered by the furthest 10 cells migrating from the center well towards the chemotactic factor (A) and that found for similar cells on the opposite facing control medium on the opposite site (B) were also used to score the chemotactic index (A/B) defined by Nelson.

We have completed a large number of pilot studies with this technique. While these need not be presented in detail, the data clearly indicate that this procedure can be effectively used to study chemotaxis for granulocytes, eosinophiles, macrophages, lymphocytes, and mixed cellular populations. Since they provide for sustained gradients which permit significant linear cell movement over protracted

time intervals; (2) permit simultaneous comparison of both random and directional movement; (3) yield estimates of the total number of migrating cells; and (4) permit adequate morphologic identification of the cells; these plates appear to be a definite advance over the Boyden chamber.

Examples of our studies on lymphocyte migration are shown in the stem-leaf plot (Table XX) and photomicrographs (Table XXI). This data demonstrates that more lymphocytes are found further from the edge of the center well along a line which connects the centers of the middle well and an outer well containing endotoxin activated serum than are present on a comparable sample moving in the opposite direction towards culture media. This yields a chemotactic index of 3 (A/B) and a chemotactic differential of 132μ ($A-B$) by the Nelson criteria. Further, stem-leaf plot illustrates that many more cells are moving towards the endotoxin activated sera. Such findings certainly suggest that $C'3a$, $C'5a$, or other unknown products being formed in the outer well are truly chemotactic for lymphocytes. It should also be emphasized that this data also demonstrates that both random and directional migration of lymphocytes can be observed under agarose. When similar studies were repeated using serum from rats with macroglobulinemia, endotoxin activation resulted in dramatic movement of normal thoracic duct lymphocytes towards this well while virtually no random migration was found in the opposite direction. By Nelson's criteria, these plates show a chemotactic index of 14 and a chemotactic differential of 858μ . Since careful histologic examination of these plates proved that all of the migrating cells were lymphocytes and we have thus far been unable to show that either granulocytes or macrophages show similar directional movement to endotoxin-activated macroglobulin sera, this may well be selective lymphocyte chemotaxis to an IgM split product similar to that described by Higuchi, et al. This selectivity is made even more interesting when compared with observations on the movement of malignant lymphocytes shown in Table XX. These cells isolated from the peripheral blood during the leucosarcoma stage after implanting a syngeneic lymphoma display the typical morphologic features of large, immature lymphocytes in Wright's and Giemsa stained preparations. When studied *in vitro* they exhibit marked random migration under agarose but no evidence of specific chemotactic response to either normal or macroglobulin activated serum. We believe that these findings document the validity of our methodology and provide a foundation for initiating an intensive effort to study the mechanisms of lymphocyte chemotaxis as proposed in our request for contract renewal.

Another potential use of this technique is shown in Table XXII where the effects of Cytochalasin on random lymphocyte migration under agarose are illustrated. These results establish that the dose of Cytochalasin A required to totally suppress lymphocyte motility is at least 50% greater than that reported in the literature. This technique then appears to offer a more stringent means for assaying the pharmacologic inhibition of cellular motility than have been available in the past.

PROJECT V. Studies of the Alterations in Regional Lymph Nodes after Stimulation with Antigens and Adjuvants.

Evidence summarized in our previous annual progress reports provides strong support for the concept that continual recirculation of long-lived lymphocytes through organized lymphatic tissue provides an efficient form of immunosurveillance facilitating both antigen recognition and cell collaboration in the developing immune response. The belief that lymphocyte recirculation might enhance immunity by permitting preferential sequestration of antigen reactive cells within antigen-stimulated nodes is as old as the concept of recirculation, and has finally been supported by radiokinetic studies in several different laboratories. Our own work has certainly supported this thesis and helped delineate some of the mechanisms which regulate this response. In the past year, we have completed additional studies which have begun to correlate sequential microvascular changes, altered structure of the cortex and medulla, with lymphocyte traffic patterns and cellular interactions at sequential stages in the evolving immune response to known antigens. Perhaps the simplest model system studied has been the regional lymph node in Lewis rats draining sites of skin grafting with AgB incompatible skin - where a short lived antigenic burst results in both humoral and cellular immunity. Data compiled from studying 42 rats at time intervals ranging from before to 28 days after grafting by combined microvascular perfusion, light and electron microscopy, autoradiography, and radiokinetic techniques are summarized in Table XXIII. These results show that up to 50% differences in the total uptake of radioactivity can be observed in axillary nodes of normal rats which show roughly comparable size, vascular anatomy, and morphology. However, in all rats examined between one and 28 days post-grafting, the traffic index derived by dividing the total number of organ counts within the regional lymph node by that formed in the contralateral node consistently ranged between two to six - a difference which was significant by standard analysis ($P = .005$). Attempts to relate this accumulation of radiolabel to morphologic changes were quite interesting in that the early rise in organ uptake correlated well with increase in nodal weight, elevation of the LMI, and morphologic changes of altered vascular permeability and the formation of cellular aggregates within the lymphatic sinuses. This early increase in radioactivity almost certainly reflects increased blood flow with delivery of more labeled cells to the node since our sequential analysis of the regional versus contralateral node uptake of labeled cells showed that the regional node always contained two to eight times more radioactivity - even in the first one-half to two hours of infusion which is the shortest transit times known for lymphocytes moving across the node (see Project II). This finding effectively excludes "trapping" due to impaired egress as a major contributor to the increase in nodal size. Further, our autoradiographic studies failed to provide any evidence for "hold up" of radiolabeled cells within the nodal parenchyma or sinuses over this time span. These cellular plugs rapidly cleared within two to three days, and the increased LMI and cortical expansion

disappeared within one week. The node then evolved to a relatively normal appearance save for a slightly prominent medulla and the presence of several large germinal centers. Despite this morphologic evidence for return of normal cell traffic patterns as the B cell humoral immune responses came into the fore, the radiokinetic traffic index continued to show that four times as much label accumulated within the enlarged, stimulated node than were found on the smaller contralateral side. This can best be explained by vascular proliferation with concomitant increased blood flow into these nodes as shown by studies described in our previous progress reports. The biological usefulness of this enhanced traffic of recirculating cells into a lymph node when the antigenic stimulus is ceasing and a full immune response has evolved must be questioned, but it may be that such altered cellular traffic continues to supply the progenitors for clonal bursts of cellular proliferation to sustain the humoral antibody response or facilitates entry of suppressor cells into the node.

These questions raised the issue as to whether we could alter this relatively simple pattern of evolutionary changes within the node by sustained or repetitive antigenic stimulation. The model selected for use here was a methyl cholanthrene induced sarcoma which is syngeneic for Lewis rats and still possesses relatively potent tumor specific transplantation antigens which are immunogenic to the host. Since this sarcoma does not metastasize to the regional nodes, yet provides a sustained bolus of antigen due to rapid membrane turnover and focal necrosis within the control tumor mass, it was thought to be suitable for our purpose. Data summarized in Table XXIV demonstrates that the regional nodes draining such tumors show virtually the same changes in morphology, microcirculation, and cell traffic as those seen in the skin graft model for the first week. Then, these nodes progress to marked sinus histiocytosis with progressive expansion of the medullary cords at the expense of a thinned cortex which becomes dominated by numerous germinal centers. Cellular traffic continues through these nodes with the same general increase seen in the skin graft model, and there are no changes to indicate that this suppresses the massive B cell response occurring locally within the node. While the humoral antibodies produced in this tumor system fail to suppress tumor growth, the overall evolution of a massive B cell response within these nodes is really quite intriguing for this is precisely what we may wish to achieve in our attempts to achieve immunopotential against biologic agents with adjuvants. Indeed, our preliminary observations on regional nodes stimulated with pertussis vaccine and peanut oil as adjuvants suggest that both agents can produce changes mimicking those described above.

PRESENTATIONS

- (1) Lipsky, J., Anderson, N.D. and Leitman, P.L.: Further studies on the immunosuppressive effects of D and L chloramphenicol. (Presented at National Meeting of the Pharmaceutical Manufacturers Association, November, 1976).
- (2) Lipsky, J., Anderson, N.D., and Leitman, P.L.: Suppression of erythropoiesis by D and L chloramphenicol. (Presented at International Congress of Antibiotics and Chemotherapy, November, 1976).
- (3) Leitman, P.L., Lipsky, J. and Anderson, N.D.: Mechanisms responsible for the lymphohemopoietic alterations induced by chloramphenicol. (Presented at the Pharmacology Guest Lectureship at Duke University in December, 1976).
- (4) Anderson, N.D.: A new look at lymphocyte recirculation. (To be presented at the University of Pennsylvania School of Veterinary Medicine in May, 1977).
- (5) Anderson, N.D. and Anderson, A.O.: Cellular interactions which regulate lymphocyte recirculation. (To be presented at the International Congress of Immunology in Sidney, Australia in July, 1977).

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1. Anderson, N.D., Anderson, A.O. and Wyllie, R.G.: "Specialized Structure and Metabolic Activities of High Endothelial Venules in Rat Lymph Nodes". Immunol. 31:455, 1976.
2. Anderson, A.O. and Anderson, N.D.: "Lymphocyte Emigration from High Endothelial Venules in Rat Lymph Nodes". Immunol. 31:731, 1976.
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5. Bell, W.R., Anderson, N.D. and Anderson, A.O.: "Heparin-Induced Coagulopathy". J. Lab. Clin. Med. (In Press).
6. Anderson, N.D. and Anderson, A.O.: "Cellular Interactions Which Regulate Lymphocyte Recirculation". Int. Cong. Immunol. (Accepted for Publication).
7. Anderson, N.D., Wyllie, R.G., Frost, J.L. and Shaker, I.J.: "Endothelial Injury in Rejecting Rat Renal Allografts". Am. J. Path. (Accepted for Publication).
8. Anderson, N.D., Wyllie, R.J. and Shaker, I.J.: "The Pathogenesis of Endothelial Injury in Rejecting Rat Renal Allografts". (Submitted for Publication).
9. Anderson, N.D. and Weiss, L.: "The Role of Splenic Lymphatics in Lymphocyte Recirculation". (Submitted for Publication).

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TABLE I

LYMPHATIC TISSUE WEIGHT IN NORMAL LEWIS RATS AT VARYING AGES

<u>Type of Node</u>	<u>30 - 40 g. Rats</u> <u>Weight</u>		<u>% Total Lymph Node Mass</u>
	<u>Mean</u>	<u>Range</u>	
OTH	49	(37 - 69)	11.6
MES	145	(117 - 205)	34.2
AB	76	(37 - 120)	17.8
SM	48	(27 - 74)	11.4
PP	108	(79 - 146)	25.0
TOTAL	426		-
Spleen	239	(181 - 312)	-

	<u>80 - 90 g. Rats</u> <u>Weight</u>		<u>% Total Lymph Node Mass</u>
	<u>Mean</u>	<u>Range</u>	
OTH	134	(85 - 177)	18.8
MES	229	(166 - 290)	32.2
AB	108	(76 - 141)	15.2
SM	105	(77 - 174)	14.8
PP	135	(98 - 164)	19.0
TOTAL	711		-
Spleen	482	(418 - 584)	-

	<u>190 - 200 g. Rats</u> <u>Weight</u>		<u>% Total Lymph Node Mass</u>
	<u>Mean</u>	<u>Range</u>	
OTH	114	(78 - 132)	15.2
MES	187	(148 - 258)	24.9
AB	135	(95 - 200)	18.0
SM	134	(81 - 174)	17.8
PP	180	(146 - 240)	24.0
TOTAL	750		-
Spleen	600	(444 - 727)	-

TABLE II

THE PATTERN OF ACCUMULATION OF RADIOACTIVITY IN BODY ORGANS OF 200 g. RATS AT SEQUENTIAL INTERVALS AFTER IV INFUSION WITH 300×10^6 VIABLE ^3H -URIDINE LABELED THORACIC DUCT LYMPHOCYTES

% of Total Dose ($\frac{\text{DPM/organ}}{\text{DPM injected}}$) Accumulating in Various Organs										
Time after Injection	Lymph Nodes		Spleen		Liver		Lungs		Gut	
	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)	Mean	(Range)
10 min.	0.5	(0.3-0.7)	5.0	(4 - 7)	19	(14 -26)	48.3	(44-52)	0.4	(0.3- 1.2)
30 min.	1.83	(1.4-2.0)	41.3	(36-50)	22.3	(17 -27)	30	(20-47)	3.1	(1.5- 4.0)
1 hr.	2.3	(2.1-3.4)	58.0	(54-63)	20.0	(17 -25)	11.3	(10-13)	2.7	(2.1- 3.4)
2 hr.	3.2	(2.8-3.8)	35.5	(32-40)	11.2	(9 -13)	4.1	(3- 5)	2.3	(1.5- 3.2)
3 hr.	3.3	(2.1-4.5)	28.2	(24-33)	6.6	(6 -7.3)	1.0	(.8-1.2)	3.8	(3.3- 4.8)
4 hr.	4.6	(4.0-5.6)	31.1	(24-36)	12.6	(5 -15.7)	1.9	(1.0-3.2)	8.9	(5.8-11.9)
8 hr.	6.5	(5.2-8.0)	17.2	(12-20)	11.5	(8 -12)	1.5	(0.9-2.0)	8.3	(5.9-10.8)
18 hr.	16.1	(11 - 22)	8.3	(4.3-13.0)	6.0	(4 - 7)	0.6	(0.5-0.9)	2.3	(1.6- 3.2)
24 hr.	13.2	(9 - 16)	3.5	(3.0- 4.0)	6.4	(5.7- 7.3)	0.6	(0.1-1.1)	6.7	(3.0- 7.3)

TABLE III

THE ACCUMULATION OF RADIOACTIVITY IN BODY ORGANS AFTER INFUSING 300×10^6 VIABLE ^3H -URIDINE-LABELLED THORACIC DUCT LYMPHOCYTES INTRAVENOUSLY INTO 80 g. RATS

Time after Injection	% of Total Dose ($\frac{\text{DPM/organ}}{\text{DPM injected}}$) Accumulating in Various Organs		Spleen	Liver	Lungs	Gut
	Lymph Nodes					
	Mean	(Range)	Mean	(Range)	Mean	(Range)
30 min.	3.60	(2.28-3.93)	32.3	(29-35)	21.3	(15-28)
2 hr.	6.1	(6 -7.5)	17.5	(16-20)	24.0	(22-26)
4 hr.	5.8	(4.9 -6.3)	20.4	(18-23)	9.9	(8-12)
8 hr.	8.8	(6.1-11.7)	10.9	(6-15)	7.2	(4- 9.6)
24 hr.	13.3	(11-15.7)	8.2	(3-14)	4.0	(3- 4.4)

TABLE IV

THE ACCUMULATION OF RADIOACTIVITY IN BODY ORGANS AFTER INFUSING
300 x 10⁶ VIABLE ³H-URIDINE LABELED THORACIC DUCT LYMPHOCYTES INTO 30 g. RATS

Time after Injection	% of Total Dose ($\frac{\text{DFM/organ}}{\text{DFM injected}}$) Accumulating in Various Organs			
	Mean	Mean	Mean	Mean
$\frac{1}{2}$ hr.	1.7 (1.4-1.8)	15.2 (14-17)	29.1 (24 -33)	32.3 (29-36)
2 hr.	2.53 (2 -3.1)	23.7 (22-25)	15.6 (15 -16.4)	7.1 (6.2-8.1)
4 hr.	2.10 (1.8-2.3)	12.1 (11-13.8)	7.3 (6.8- 8.0)	2.1 (1.4-2.8)
8 hr.	4.4 (4.1-4.8)	13.5 (12.8-13.9)	5.4 (5 - 5.6)	1.7 (1.3-1.9)
24 hr.	3.8 (3 -4.7)	2.8 (2.6- 3.0)	5.4 (4.3- 6.5)	1.1 (.9-1.2)
				9.1 (8 -10.5)

TABLE V

ORGAN DISTRIBUTION OF RADIOACTIVITY IN LEWIS RATS BEARING ESTABLISHED
THORACIC DUCT FISTULAE FOLLOWING IV INFUSION WITH 300×10^6 ^3H -URIDINE-LABELLED LYMPHOCYTES

Time after Injection	Thoracic Duct		Lymph Nodes		Spleen		Liver		Lungs		Gut	
	Lymph		Lymph		Mean		Mean		Mean		Mean	
	Mean	(Range)	Mean	(Range)	(Range)	(Range)	(Range)	(Range)	(Range)	(Range)	(Range)	(Range)
2 hr.	0.3	(.008-.04)	0.85	(.65-1.70)	18.5	(16.7-20.3)	14.2	(9.2-19.0)	7.1	(4.0-10.2)	2.3	(1.6-3.8)
4 hr.	.1	(.08-.12)	2.73	(2.0-3.4)	15.8	(8.4-23.3)	17.7	(15-20.1)	1.89	(1.4-2.1)	5.2	(4.5-5.6)
6 hr.	.3	(.18-3.4)	--	--	--	--	--	--	--	--	--	--
8 hr.	.3	(.20-3.6)	4.3	(4.0-4.5)	15.3	(11.1-17.6)	5.7	(6.1-8.2)	2.4	(1.2-4.1)	4.1	(2.6-6.6)
18 hr.	14	(10-28)	4.23	(2.9-7.5)	3.3	(0.8-3.8)	5.0	(4.3-6.0)	0.6	(0.4-0.8)	9.6	(1.2-11.7)

% of Total Dose Injected (DPM in organ or lymph)
Total DPM injected

Tissue or Blood Fluid Examined

TABLE VI

THE ACCUMULATION OF RADIOACTIVITY IN PERIPHERAL LYMPH NODES OF SPLENECTOMIZED RATS AFTER INTRAVENOUS INFUSION WITH 300×10^6 VIABLE ^3H -URIDINE-LABELED THORACIC DUCT LYMPHOCYTES.

% of Total Dose ($\frac{\text{DPM/organ}}{\text{DPM injected}}$) Accumulating in Different Organs #

Time after Injection	Lymph Nodes						Liver	Lungs	Gut
	OTH	MES	PP	AB	SM	Total			
1 hr.	1.45	2.6	1.3	1.3	.5	7.5	22.1	18.0	8.5
2 hr.	2.0	3.8	1.95	1.5	1.2	10.45	19.8	9.6	6.8
3 hr.	2.5	10.1	3.2	1.5	1.1	18.40	16.1	11.4	12.1
4 hr.	4.4	6.8	2.9	1.5	1.2	16.80	15.5	7.1	9.6
8 hr.	6.0	9.6	1.9	4.9	2.7	25.10	11.1	3.1	8.7
18 hr.	5.2	11.3	1.9	5.1	2.9	26.4	8.0	1.1	8.6

Each Value Represents the Mean Value Derived from Studying 3-5 animals.

TABLE VII

THE ACCUMULATION OF RADIOACTIVITY IN BODY ORGANS OF RATS
INFUSED WITH ^3H -URIDINE LABELED TDL ONE WEEK AFTER
IV CHALLENGE WITH 3×10^8 SHEEP ERYTHROCYTES

Body Weight		Mean Lymphatic Tissue Weight (mg.)					
Mean	Range	OTH	MES	AB	SM	PP	Spleen
198	(190-210)	222	420	225	199	217	857

% of Total Dose ($\frac{\text{DPM/organ}}{\text{DPM injected}}$) Accumulating in Different Organs #

Time after Injection	Lymph			Nodes		Total	Spleen	Liver	Lungs
	OTH	MES	PP	AB	SM				
$\frac{1}{2}$ hr.	0.7	2.2	0.6	0.6	0.7	4.80	25.1	26.5	17.2
2 hr.	0.6	2.2	0.4	0.5	0.6	4.30	25.3	16.7	3.33
4 hr.	0.7	2.0	.4	0.7	0.5	4.30	14.8	11.4	2.20
8 hr.	2.0	2.8	.8	1.1	1.4	8.10	10.9	6.23	1.99
18 hr.	2.0	8.3	0.2	1.8	2.8	15.60	3.7	5.53	0.86

Mean Values for Derived from Studying 3-5 rats at each time interval.

TABLE VIII

THE ACCUMULATION OF RADIOACTIVITY IN BODY ORGANS OF RATS
BEARING ESTABLISHED LYMPHOMA AFTER INFUSING
³H-URIDINE LABELED LYMPHOCYTES

Body Weight		Mean Weight of Lymphatic Tissue (mg.)					
Mean	(Range)	OTH	MES	AB	SM	PP	Spleen
154	(140 - 180)	135	230	108	105	135	1280

% of Total Dose ($\frac{\text{DPM/organ}}{\text{DPM injected}}$) Accumulating in Different Organs #

Time after Injection	Lymph Nodes						Spleen	Liver	Lungs
	OTH	MES	AB	SM	PP	Total			
$\frac{1}{2}$ hr.	.37	1.60	.44	.26	.73	3.40	32.3	21.3	11.0
2 hr.	.96	3.08	.44	.37	1.49	6.34	22.9	24.0	3.1
4 hr.	1.00	2.70	.39	.52	1.22	5.83	20.4	9.9	2.2
8 hr.	.94	3.50	2.60	.85	.97	8.86	10.8	7.2	1.3
18 hr.	1.57	5.60	3.50	2.24	.49	13.40	9.0	4.0	1.0

Mean values derived from studying 3-5 rats at each time interval.

TABLE IX

SPECIFIC ACTIVITY (DPM/mg. TISSUE) OF VARIOUS LYMPH NODES
AFTER INFUSION WITH 300×10^6 LABELED LYMPHOCYTES

<u>Time after Injection</u>	<u>Other</u>	<u>Mesenteric</u>	<u>Axillary- Brachial</u>	<u>Submandibular</u>	<u>Peyer's Patches</u>
$\frac{1}{2}$ hr.	90	151	75	85	154
1 hr.	132	241	113	140	149
2 hr.	68	247	72	101	218
4 hr.	176	235	168	162	147
8 hr.	300	402	215	351	139
18 hr.	580	1,164	697	932	107
24 hr.	505	924	624	702	147

TABLE X

THE ACCUMULATION OF RADIOLABELED LYMPHOCYTES IN RATS AFTER
EXCISION OF THE MESENTERIC NODES, SMALL INTESTINES (GALT), AND SPLEEN

<u>Time after Injection</u>	% of Total Dose Injected in Various Organs					
	Remaining Lymph Nodes		Liver		Lungs	
	<u>Mean</u>	<u>(Range)</u>	<u>Mean</u>	<u>(Range)</u>	<u>Mean</u>	<u>(Range)</u>
2 hr.	2.9	(1.9-4.0)	27	(26 -29)	15	(12 -18)
4 hr.	3.0	(2.3-3.6)	27	(20 -32)	8.8	(6 -13)
8 hr.	1.3	(1.1-1.6)	7	(5.5- 8.0)	5.7	(4.5- 6.6)
18 hr.	.8	(.5- .8)	5.6	(5.4- 6.0)	2.4	(2.2- 2.7)

TABLE XI

THE ACCUMULATION OF RADIOACTIVITY IN THE ORGANS OF RATS
SUBJECTED TO SELECTIVE EXCISION OF THE MESENTERIC LYMPH NODES
BEFORE INFUSING 300×10^6 URIDINE-LABELED THORACIC DUCT LYMPHOCYTES

% of Total Dose ($\frac{\text{DPM /organ}}{\text{DPM injected}}$) Accumulating in Different Organs #

Time after Injection	Lymph			Nodes		Total	Spleen	Liver	Lungs
	OTH	MES	PP	AB	SM				
2 hr.	1.07	-	0.37	0.60	0.6	2.64	24.5	15.4	5.7
4 hr.	1.70	-	0.81	0.87	0.3	3.68	29.5	6.8	1.3
8 hr.	3.13	-	0.97	1.80	.67	6.57	17.8	6.1	1.4
24 hr.	7.13	-	0.97	4.33	1.10	13.53	7.4	5.7	0.8

Each point represents the mean value derived from studying 3-4 rats at each time interval.

TABLE XII

DISTRIBUTION OF LABELED LYMPHOCYTES WITHIN DIFFERENT LYMPH
NODES AT SEQUENTIAL TIME INTERVALS AFTER INFUSION WITH
 3×10^8 ^3H -URIDINE-LABELED THORACIC DUCT LYMPHOCYTES

Time after Injection

	Number of Cells per Anatomical Location				
	HEV Lumen	HEV Wall	Cortex (10HpF)	Sinuses	Total (10HpF)
Submandibular Node					
3 min.	10.0	15.8	1.0	0.2	27.0
10 min.	4.4	28.4	16.4	0	49
30 min.	1.8	27.8	28.8	1.2	59.6
120 min.	0.4	12.0	150.8	10.2	173.4
Axillary Node					
3 min.	8.0	10.2	3.6	0	21.8
10 min.	11.2	22.8	15.8	0	53.6
30 min.	4.8	38.6	22.0	.4	65.4
120 min.	2.4	29.0	72.1	0	103.3
Mesenteric Node					
3 min.	12.8	25.8	1.2	0	39.8
10 min.	11.0	49.4	42.6	2.0	109.6
30 min.	1.6	16.6	77.8	7.6	103.6
120 min.	0.4	22.2	258.8	15.6	287.0

TABLE XIII

THE ACCUMULATION OF RADIOACTIVITY IN THE ORGANS OF NORMAL RATS
FOLLOWING IV INFUSION WITH 300×10^6 ^3H -URIDINE
LABELED MALIGNANT LYMPHOCYTES

% of Total Dose ($\frac{\text{DPM/organ}}{\text{DPM injected}}$) Accumulating in Different Organs #

<u>Time after Injection</u>	<u>Lymph Nodes</u>	<u>Spleen</u>	<u>Liver</u>	<u>Lungs</u>	<u>Gut</u>	<u>Blood</u>
$\frac{1}{2}$ hr.	0.1	6.82	29.7	47.2	1.63	1.82
1 hr.	0.1	7.28	27.1	26.4	1.78	3.82
2 hr.	0.2	5.61	23.3	19.6	3.71	9.71
4 hr.	0.2	6.22	29.5	14.7	4.51	6.50
8 hr.	0.4	7.07	22.2	5.2	5.12	5.06
18 hr.	0.5	5.85	12.12	2.36	5.38	5.40

Each value represents the mean derived from studying 3-5 rats.

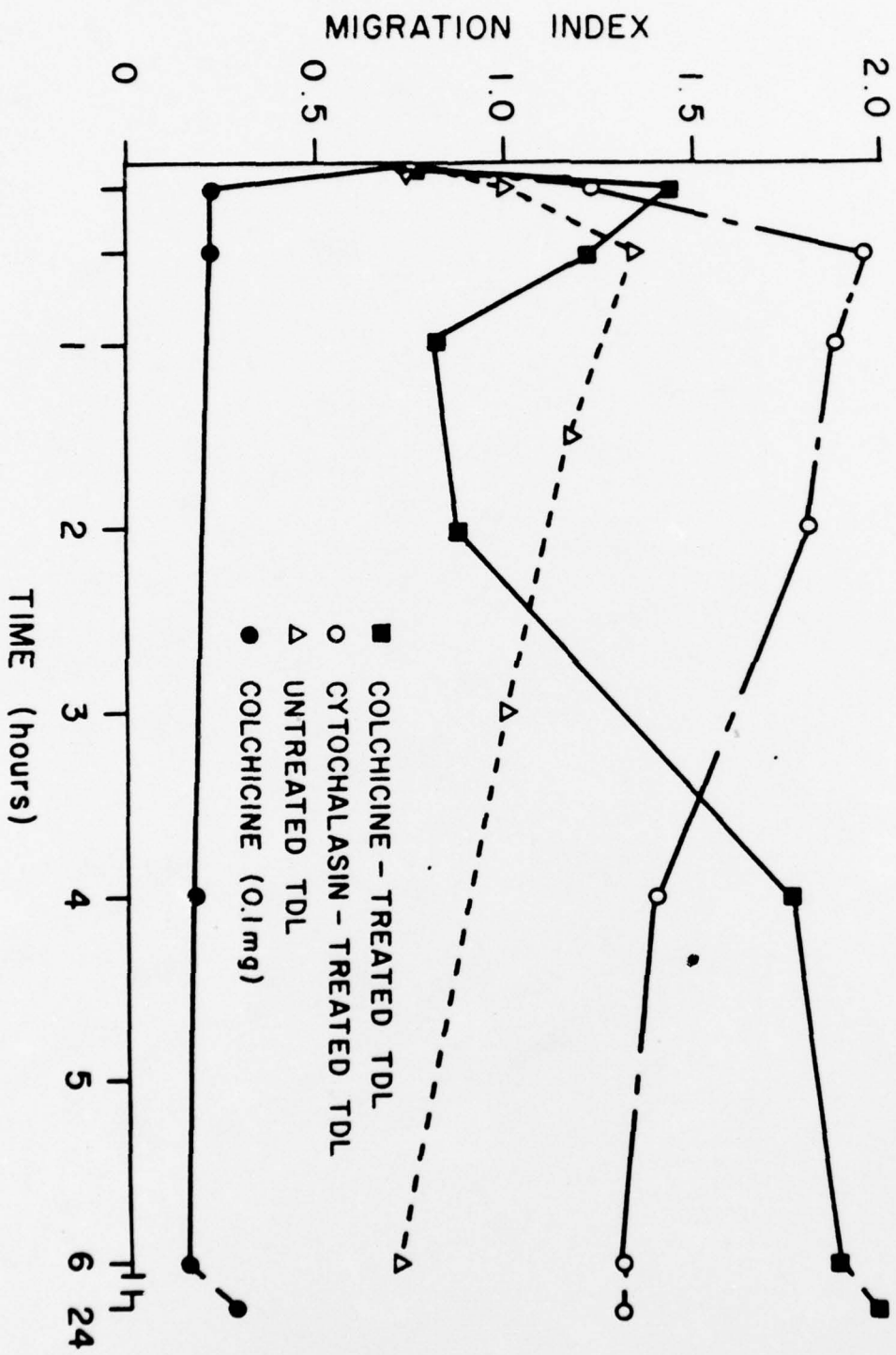
TABLE XIV

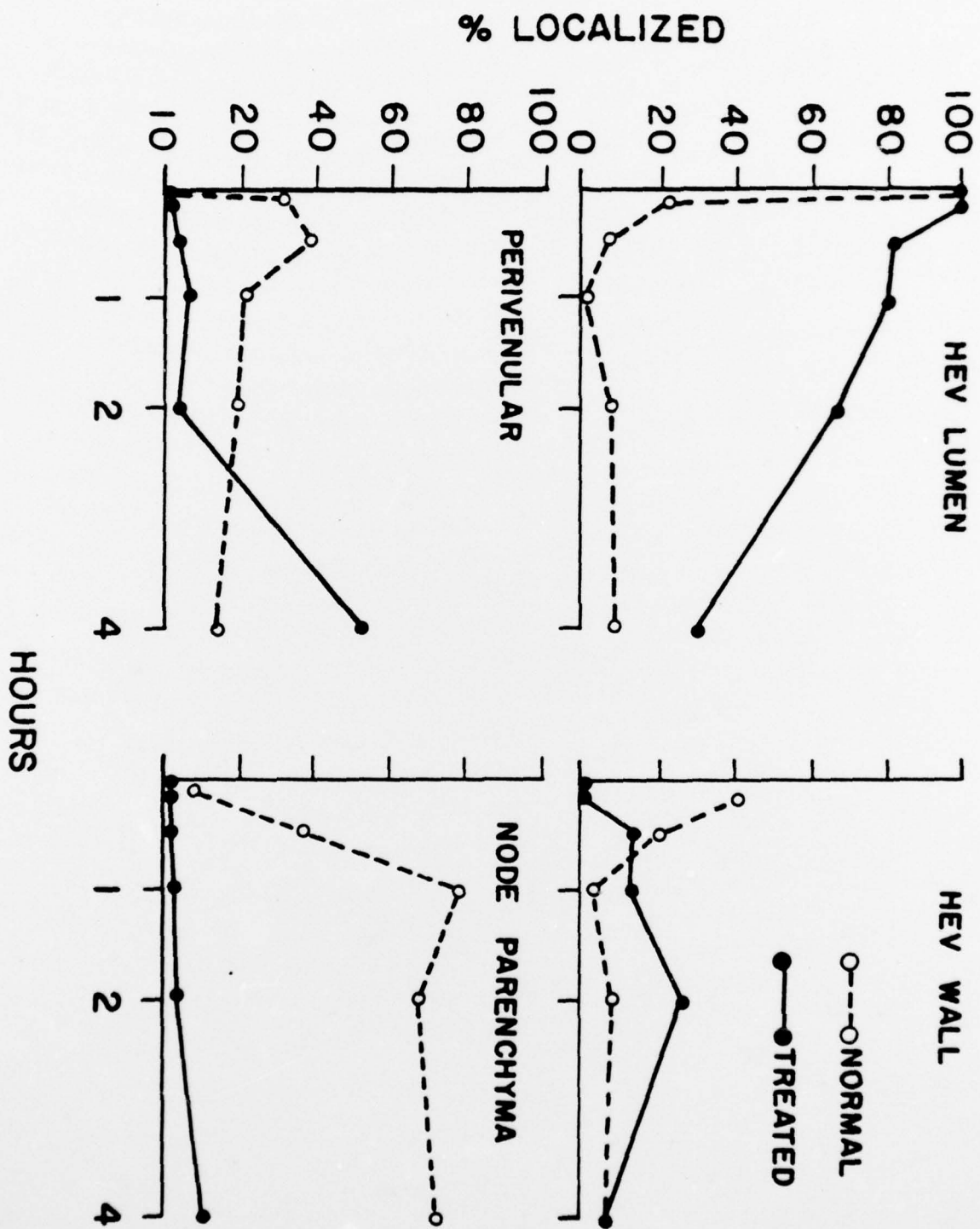
THE ACCUMULATION OF RADIOACTIVITY IN RAT ORGANS
FOLLOWING THE INFUSION OF 300×10^6 CYTOCHALASIN OR
HEAT-KILLED THORACIC DUCT LYMPHOCYTES

% of Total Dose Injected ($\frac{\text{DPM/organ}}{\text{DPM injected}}$)

Time after Injection	Cytochalasin Cells				Heat-Killed Cells			
	Lymph Nodes	Spleen	Liver	Lungs	Lymph Nodes	Spleen	Liver	Lungs
10 min.	0.1	18.3	18	28.2	--	--	--	--
30 min.	0.1	25	20.3	14	.3	1.6	18.4	10.2
1 hr.	0.12	17	10	9.3	.46	0.7	15.6	5.3
2 hr.	0.07	23	13.3	4.0	--	--	--	--
3 hr.	0.09	14.3	13.6	2.3	--	--	--	--
4 hr.	0.14	3.6	9.3	1.0	1.6	2.1	24.6	3.16
8 hr.	0.14	4.0	7.0	0.6	1.83	2.0	21.6	1.9
18 hr.	0.13	2.1	7.3	0.2	1.4	1.2	18.0	1.5
24 hr.	0.15	2.0	7.5	0.1	--	--	--	--

TOTAL LYMPHOCYTE MIGRATION INTO LYMPH NODES FOLLOWING I.V. INFUSION OF TDL, PRETREATED OR COLCHICINE





EFFECT OF CYTOCHALASIN-A TREATMENT ON LYMPHOCYTE RECIRCULATION

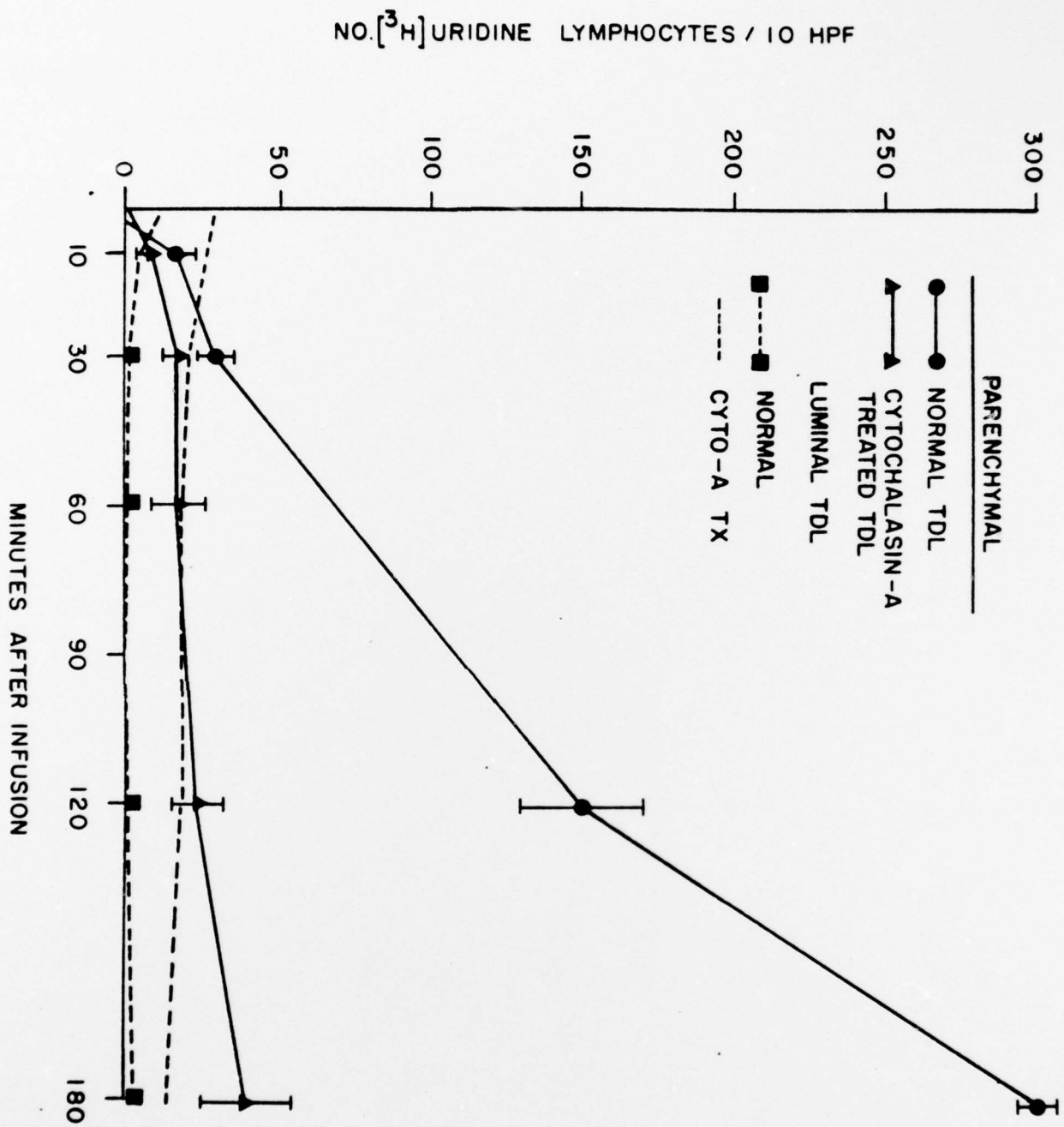


TABLE XVIII

THE ACCUMULATION OF RADIOACTIVITY IN THE ORGANS OF RATS INFUSED WITH RADIOLABELED LYMPHOCYTES INCUBATED WITH COLCHICINE IN VITRO

% of Total Dose ($\frac{\text{DPM/organ}}{\text{DPM injected}}$) Accumulating in Different Organs #

<u>Time after Injection</u>	<u>Lymph Nodes</u>	<u>Spleen</u>	<u>Liver</u>	<u>Lungs</u>	<u>Gut</u>	<u>Blood</u>
$\frac{1}{2}$ hr.	2.1	28.3	25.2	29.6	3.0	2.4
2 hr.	2.4	24.5	14.9	5.6	2.7	2.0
4 hr.	4.1	30.1	6.2	1.2	3.2	3.8
8 hr.	7.3	16.8	6.3	1.4	9.1	4.2
24 hr.	14.6	6.2	5.1	0.8	6.5	5.1

Each value represents the mean of three animals studied at each time interval.

TABLE XIX

THE ACCUMULATION OF RADIOACTIVITY IN THE ORGANS OF RATS
INFUSED WITH ^3H -URIDINE LABELED TDL ONE HOUR AFTER
IP INJECTION WITH COLCHICINE (1mg./kg.)

% of Total Dose ($\frac{\text{DPM/organ}}{\text{DPM injected}}$) Accumulating in Different Organs #

<u>Time after Injection</u>	<u>Lymph Nodes</u>	<u>Spleen</u>	<u>Liver</u>	<u>Lungs</u>	<u>Gut</u>	<u>Blood</u>
$\frac{1}{2}$ hr.	0.8	5.5	17.7	38.9	2.8	2.1
2 hr.	0.6	5.7	18.3	15.7	5.1	2.6
4 hr.	0.9	9.3	17.0	8.6	6.1	5.7
8 hr.	2.2	10.3	14.3	3.4	7.7	6.4
24 hr.	6.7	4.3	8.8	0.9	8.4	8.4
24 hr. after 2nd dose given IP at 8 hours.	2.1	2.9	6.3	1.2	4.9	9.8

Each value represents the mean of 3-4 animals studied at each time interval.

TABLE XX - (a)

STEM AND LEAF PLOTS: FARTHEST TEN CELLS WITHIN 66 MICRA OF AXIS BETWEEN WELLS

Arbitrary Units (66 μ) Toward Attractant	Thoracic Duct Lymphocytes to Endotoxin Activated Serum Not Using Macroglobulin	Thoracic Duct Lymphocytes to Endotoxin Activated Serum Using Macroglobulin	LY8 Tumor Cells to Endotoxin Activated Serum using Macroglobulin
38.			
37.			
36.			
35.			7
34.			
33.			772
32.			83
31.			54321
30.			3
29.			885520
28.			8
27.			32
26.			
25.			70
24.			97322
23.			432
22.			77666531000
21.			870
20.			4
19.			2
18.			
17.			71
16.			9
15.		0	
14.		2	
13.		31	
12.		944	
11.		8863222	
10.		66533	
9.		77776653322	
8.		87765544432110	
7.		420	
6.		933	
5.		876443	
4.		731	
3.	9863222	8	
2.	74400		
1.	98773		
+0.	8755422		

TABLE XX - (b)

STEM AND LEAF PLOTS: FARTHEST TEN CELLS WITHIN 66 MICRA OF AXIS BETWEEN WELLS

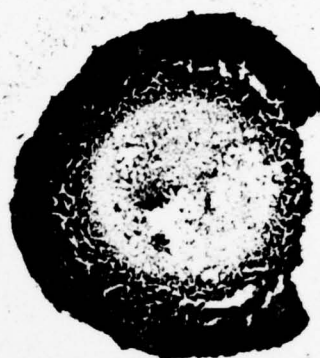
Arbitrary Units (66 μ) Away from Attractant	Thoracic Duct Lymphocytes to Endotoxin Activated Serum Not Using Macroglobulin	Thoracic Duct Lymphocytes to Endotoxin Activated Serum Using Macroglobulin	LY8 Tumor Cells to Endotoxin Activated Serum Using Macroglobulin
-0.	544		
1.	510	6	
2.		11	
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			99877
12.			91
13.			6000
14.			76621
15.			886542
16.			75400
17.			3
18.			995532
19.			64
20.			86
21.			5
22.			0
23.			3
24.			
25.			7
26.			866
27.			3
28.			9
29.			5
30.			
31.			32
32.			.
33.			
34.			
35.			

LEGENDS FOR FIGURE XXI

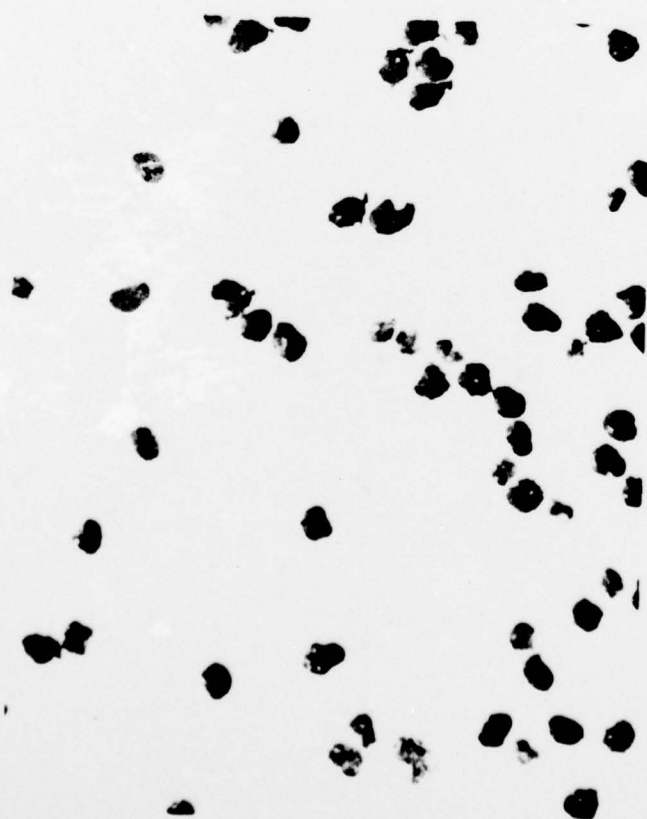
- A. Low power photomicrograph of center well in chemotactic assay showing directional migration of cells towards outer well containing endotoxin-activated serum (edge just visible). Note the absence of cellular movement in the opposite direction towards well containing control medium.
- B. Low power photomicrograph of center well using malignant lymphocytes in chemotactic assay. Note equal dispersion of cells about the center well indicative of random migration under agarose.
- C. High power photomicrograph of cells shown in A. All migrating cells exhibit the typical morphology of small lymphocytes.



A



B



C

TABLE XXII

THE EFFECTS OF CYTOCHALASIN A ON LYMPHOCYTE
MIGRATION UNDER AGAROSE *

<u>Distance migrated (microns)</u>	<u>Dose of Cytochalasin (g/ml)</u>					
	0	2	4	6	8	10
	<u>Number of migrating lympho- cytes</u>	<u>Number of migrating lympho- cytes</u>	<u>Number of migrating lympho- cytes</u>	<u>Number of migrating lympho- cytes</u>	<u>Number of migrating lympho- cytes</u>	<u>Number of migrating lympho- cytes</u>
0 - 67	3620	3740	3790	450	0	0
67 - 133	3810	3980	1630	0	0	0
133 - 200	4300	4500	200	0	0	0
200 - 267	4250	4190	150	0	0	0
267 - 333	3450	3420	0	0	0	0
333 - 400	2020	1930	-	-	-	-
400 - 467	700	620	-	-	-	-
467 - 533	80	60	-	-	-	-

* All lymphocytes were incubated in varying concentrations of Cytochalasin, washed, and then placed in the center well of the agarose assay. Migration was scored after 18 hours incubation at 37° under 5% CO₂, using an ocular micrometer grid system to enumerate cells within each distance designated.

TABLE XXIII

ALTERATIONS IN THE REGIONAL NODE DRAINING SKIN ALLOGRAFTS

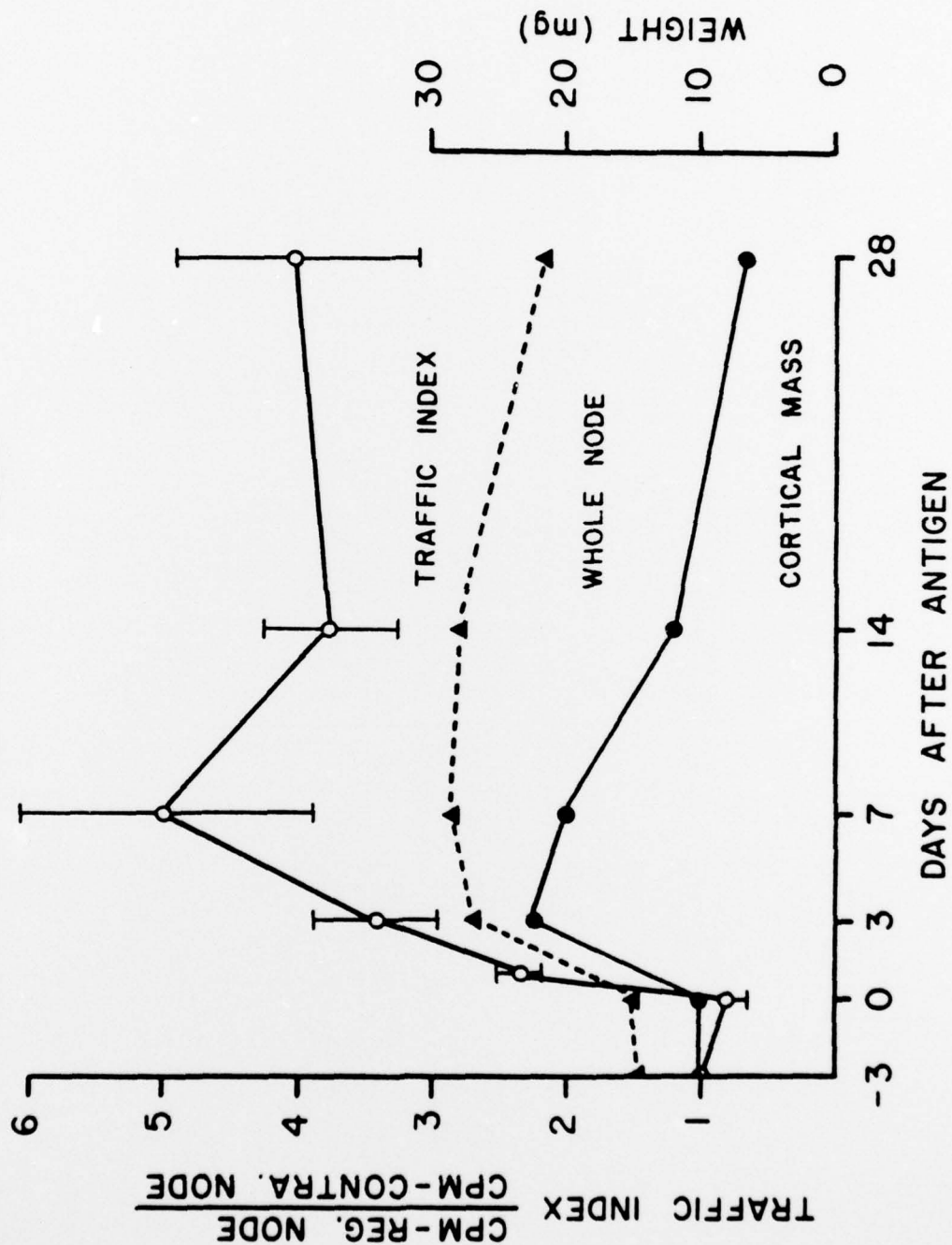
<u>Days after Grafting</u>	<u>Node Weight (mg.)</u>	<u>LMI #</u>	<u>Lymphocyte Plugs +</u>	<u>Trapping Index</u>
0	18	0.72	0	1 ± 0.5
1	21	1.14	2+	2 ± 0.2
2	25.5	1.20	2+	$3.4 \pm .6$
3	28.5	.84	+	3.8 ± 1.2
7	43.5	.97	0	6.0 ± 2.2
14	38.5	.81	0	4.1 ± 1.8
28	37.8	.68	0	4.1 ± 2.1

LMI =
$$\frac{\text{Number of migrating lymphocytes in HEV}}{\text{Number of high endothelial cells}}$$

Lymphocyte Plugs = Histologic evidence of plugging of lymph sinuses by log-jams of lymphocytes.

Trapping Index =
$$\frac{\text{Total DPM in regional node}}{\text{Total DPM in contralateral node}} \pm \text{S.E.}$$

THE EFFECT OF TST ANTIGEN ON THE LYMPH NODE



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